PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/08, A61K 35/14

A2

(11) International Publication Number: WO 97/03186

(43) International Publication Date: 30 January 1997 (30.01.97)

(21) International Application Number: PCT/US96/11640

(22) International Filing Date: 12 July 1996 (12.07.96)

(30) Priority Data:
08/501,987 12 July 1995 (12.07.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/501,987 (CIP)
Filed on 12 July 1995 (12.07.95)

(71) Applicant *(for all designated States except US)*: ACTIVATED CELL THERAPY, INC. [US/US]; 291 North Bernardo Avenue, Mountain View, CA 94043 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PESHWA, Madhusudan, Vifwauth [IN/US]; 395 Ano Nuevo Avenue, Sunnyvale, CA 94086 (US). VAN SCHOOTEN, Willem, Casper [NL/US]; 1444 Floyd Avenue, Sunnyvale, CA 94087 (US).

(74) Agents: SHOLTZ, Charles, K. et al.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

(81) Designated States: AU, CA, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR IN VITRO PROLIFERATION OF DENTRITIC CELLS, COMPOSITION CONTAINING THE CELLS ENTRAPPED IN A THREE-DIMENSIONAL MATRIX AND USE FOR IMMUNIZATION

(57) Abstract

A method of obtaining a blood-cell fraction enriched for potent antigen presenting cells is disclosed. The method includes obtaining a monocyte-depleted lymphocyte fraction, culturing the cell fraction in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the morphology of dendritic cells, harvesting non-adherent cells produced by said culturing, and enriching the portion of dendritic cells in the harvested cells by density centrifugation. Also disclosed is a PAP cell composition containing cells enriched for PAP activity in a collagen matrix.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

METHOD FOR IN VITRO PROLIFERATION OF DENTRITIC CELLS, COMPOSITION CONTAINING THE CELLS ENTRAPPED IN A THREE-DIMENSIONAL MATRIX AND USE FOR IMMUNIZATION

Field of the Invention

The present invention relates to potent antigen-presenting (PAP) cells and in particular, to methods of preparing PAP cells, methods of activating T-cells using allogeneic PAP cells matched for only one allele and to a composition containing PAP cells entrapped in a three-dimensional matrix.

References

Ausubel, F. M., *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons, Inc., Media PA.

Borrow, P., *et al.*, *J. Virol* 69:1059-1070 (1995).

Braciale, *et al.*, *Immunol. Rev.* 98:95-114

Brendel, M.D., *et al.*, *Cell Transpl.* 3:427-435 (1994).

Chao, S-H., *et al.*, *Cell Transpl.* 1:51-60 (1992).

Chehimi, J., *et al.*, In: Dendritic Cells in Fundamental and Clinical Immunology. Kamperdijk (ed). New York: Plenum Press, pp. 521-526 (1993).

Eales, L.-J., *et al.*, *Clin. Exp. Immunol.* 71:423-427 (1988).

Elovaara, I., *et al.*, *J. Exp. Med.* 177:1567-1573 (1993).

Gabrilovich, D.I., *et al.*, *Proceedings of the Amer. Soc. of Clin. Oncol.* (15 March, 1996).

Grabbe, S., *et al.*, *Immunol. Today* 16:117-121 (1995).

Guery, J.-C., *et al.*, *J. Immunol.* 154:536-544 (1995).

Hsu, F.J., *et al.*, *Nature Med.* 2:52-58 (1996).

Hu, X., *et al.*, *Cancer Res.* 56:2479-2483 (1996).

Kabel, P.J., *et al.*, *Immunobiology* 179:395-41 (1989).

Kannagi, M., *et al.*, *J. Virol.* 66:2928-2933 (1992).

Knight, S.C., *et al.*, In: Dendritic Cells in Fundamental and Clinical Immunology. Kamperdijk (ed). New York: Plenum Press, pp. 545-549 (1993).

Langer & Bacanti *Science* 260:920-926 (1993).

Macatonia, S.E., *et al.*, *Immunology* 71:38-45 (1990).

Macatonia, *et al.*, *Immunology* 74:399-406 (1991).

Markowicz, S., and Engleman, E.G., *J. Clin. Invest.* 85:955-961 (1990).

Mayordomo, J.I., *et al.*, *Nat. Med.* 1(12):1297-1302 (1995).

Mehta-Damani, A., *et al.*, *J. Immonol.* 153:996-1003 (1994).

Moore, *et al.*, *Cell* 54:777 (1988).

Mulligan, R.C. *Science* 260:926 (1993).

Nair, S., *et al.*, *J. Immunol. Meth.* 152:237 (1992).

Pope, N.M., *et al.*, *Bioconjugate Chem.*, 4:186-171 (1993).

Reddy, R., *et al.*, *J. Immunol.* 148:1585 (1992).

Rooney, C.M., *et al.*, *Lancet* 345:9-13 (1995).

Stevens, E.J., *et al.*, *J. Immunol.* 154:762-771 (1995).

Steinman, *Ann. Rev. Immunol.* 9:271-296 (1991).

Takahashi, H., *et al.*, *Int. Immunol.* 5:849-857 (1993).

Thomas R., and Lipsky, P.E., *J. Immunol.* 153:4016-40:28 (1994).

Walter, E.A., *et al.*, *New Engl. J. Med.* 333:1038 (1995).

Young, J.W., and Steinman, R.M., *Cell. Immunol.* 111:167-182 (1987).

Zhou, F., *et al.*, *J. Immunol.* 149:1599 (1992).

Zweerink, H.J., *et al.*, *J. Immunol.* 150:1763-1771 (1993).

Background of the Invention

Literature reports indicate that different researchers employ diverse and labor intensive techniques for isolation and *in vitro* generation of antigen presenting cells (APC). Not only are there various types of APC and protocols for isolating them and activating them in *vitro* cultures, but also within a given APC type, there are variations with regard to isolation and activation procedures. This is particularly true with respect to dendritic cells (DC), which can be particularly effective APC.

DC have been isolated and purified using a variety of methodologies incorporating, for example, multiple-step density-based isolations, monoclonal antibody panning and serum-supplemented cultures (Macatonia, *et al.*, 1991; Markowicz and Engleman, 1990; Young and Steinman, 1987). However, all the reported isolation procedures employ either sheep red blood cells and/or fetal calf serum, both of which contain potentially immunogenic foreign antigens and/or immunogens which can interfere with the use of the purified DC (*e.g.*, to stimulate T-cells with a selected immunogen). Furthermore, there appear to be significant differences in the characteristics of the DC purified by the various prior art methods (*e.g.*, differences in cell surface marker expression). In view of these differences, it has been proposed that there exist at least three sub-types of DC (Grabbe, *et al.*, 1995; Thomas and Lipsky, 1994), each of which may have different properties and characteristics in terms of its antigen presenting capability.

Summary of the Invention

The invention includes, in one aspect, a method for obtaining, from a human blood sample, potent antigen presenting (PAP) cells characterized by (i) a phenotype that is positive for surface antigen HLA-DR (human leukocyte antigen DR gene) and negative for surface antigens CD3, CD4, CD8, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes.

The method includes obtaining from the blood sample, a monocyte-depleted fraction containing peripheral blood lymphocytes and dendritic-precursor cells. This fraction is cultured in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the morphology of dendritic cells. Non-adherent cells are harvested, and the harvested cells are enriched for PAP cells by density centrifugation.

The fraction enriched in peripheral blood lymphocytes and dendritic-precursor cells is preferably obtained by (i) first enriching the blood sample in peripheral blood mononuclear cells by density centrifugation, and (ii) enriching the product of (i) in lymphocytes and dendritic-precursor cells by density centrifugation. The enriching in step (ii) is preferably carried out by layering the product of (i) over a separation medium having a density of 1.0650 ± 0.0010 g/mL, and an osmolarity of 300 ± 15 mOsm. The cells are then cultured in a serum-free medium. Culturing is carried out until dendritic-precursor cells undergo the desired morphological change to dendritic cells, preferably at least about 24 hours.

Exemplary serum-free media for culturing the cells containing PAP-precursor cells include Dulbecco's Modified Minimal Essential Medium (DMEM):F-12 (1:1), AIM-V, macrophage serum-free medium, nutrient-supplemented AIM-V or Enriched Monocyte SFM (available from, *e.g.*, Gibco/BRL Life Technologies, Gaithersburg, MD).

Subsequent to culture, the non-adherent cells are harvested and PAP cells are enriched by density centrifugation. The final enriching step is preferably carried out by layering the cultured cells over a separation medium having either (i) a density of 1.0550 ± 0.0010 g/mL, and an osmolarity of 290 ± 10 mOsm, or (ii) a density of 1.0800 ± 0.0010 g/mL, and an osmolarity of 540 ± 25 mOsm. Potent antigen presenting activity is obtained from cells at the interface.

The method may further include, following the enriching step, contacting the cells in the PAP-enriched fraction with a solid phase conjugated with antibodies against at least one cell surface phenotype marker selected from the group consisting of CD4, CD8, CD14,

CD3, CD16, and CD20, and removing cells in the fraction which bind to the solid phase. After this final step, the dendritic cells may constitute more than 50% of the cell fraction.

The method may further include entrapping PAP cells in the enriched fraction in a three-dimensional matrix, such as a collagen-fiber matrix, to preserve the differentiation state and antigen presentation capability of the PAP cells in culture for an extended culture period.

The invention also includes a method of preparing activated T-cells specific for a selected antigen or immunogen. The method includes incubating T-cells with allogenic PAP cells pulsed with the antigen or immunogen. The PAP cells used in the method are characterized by (i) a phenotype that is positive for surface antigen HLA DR and negative for surface antigens CD3, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes in culture, and are matched, with respect to said T-cells, for at least one HLA class I or class II allele. They may be matched for only one allele, all alleles, or any combination in between, such as being mismatched for only one allele The T-cells can be, for example, naive T-cells, memory T-cells, $CD4^+$ T-cells or $CD8^+$ T-cells.

In a related aspect, the invention includes a method of immunizing a subject against a tumor or pathogen having an known tumor- or pathogen-specific antigen or immunogen. The method includes (A) pulsing allogenic PAP cells with the selected antigen or immunogen, and (B) exposing the subject's lymphocytes to the pulsed PAP cells. The PAP cells are characterized by (i) a phenotype that is positive for surface antigen HLA DR and negative for surface antigens CD3, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes in culture, and are matched, with respect to said lymphocytes, for at least one HLA class I or class II allele. The exposing step can be achieved by, for example, injecting the pulsed PAP cells into the subject, or by removing cytotoxic T lymphocytes from the subject, contacting the lymphocytes with the pulsed PAP cells *ex vivo*, and returning the contacted lymphocytes to the subject's bloodstream.

In another aspect, the invention includes a cell composition composed potent antigen presenting (PAP) cells of the type described above entrapped in a three-dimensional matrix, as exemplified by a collagen-fiber matrix.

In a related aspect, the invention includes a method of vaccinating a subject against a tumor or pathogen with a known tumor-, tissue-specific or pathogen-specific antigen or immunogen. The method includes isolating from the subject, a blood-cell fraction enriched

for potent antigen presenting (PAP) of the type described above, entrapping the PAP cells in a three-dimensional biocompatible matrix, treating the matrix-entrapped PAP cells with either an HLA-binding peptide contained within the selected tumor-, tissue-specific or pathogen-specific antigen or immunogen, and exposing the subject's cytotoxic T cells to the matrix.

The exposing step may involve injecting the cell-containing matrix into the subject, or removing cytotoxic T lymphocytes from the patient, contacting the lymphocytes with the matrix *in vitro*, and returning the contacted lymphocytes to the patient's bloodstream.

Examples of pathogen-specific antigens include, but are not limited to, viruses, bacteria, parasites, fungi, microorganisms, and portions thereof, such as specific immunogenic proteins (*e.g.*, surface proteins, surface and internal structural proteins, and immunogenic portions thereof such as human immunodeficiency virus (HIV) env, HIV gag, HIV nef, HIV pol, HIV rev, HIV tat, HIV rev, human T cell leukemia virus I (HTLV-1) tax and influenza matrix protein (IMP); particularly peptide HIV Pol 464-472 Pep (SEQ ID NO:3) and peptide HTLV-1 Tax 11-19 Pep (SEQ ID NO:1)).

Examples of tumor or tumor-associated antigens (*i.e.*, antigens selectively expressed by cancer cells) include but are not limited to p53, carcinoembryonic antigen (CEA), HER2, MART-1, p21RAS and portions thereof, including MART-1 peptide (SEQ ID NO:2). Examples of tissue-specific antigens upregulated in neoplastic transformed tissue (*i.e.*, antigens selectively expressed on or by specific tissue/cell types when that tissue or cell type becomes transformed) include prostate-specific antigens upregulated in prostate cancer, such as prostatic acid phosphatase, prostate specific membrane antigen, and prostate specific antigen.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figures 1A and 1B are fluorescence-activated cell sorting (FACS) profiles used to characterize the purity of dendritic cells (DC) in a PAP cell-enriched fraction obtained from the interface of a density centrifugation in MEP (Metrizamide Equivalent Percoll) following a two day culture of monocyte depleted peripheral blood mononuclear cells. The cells were stained with CD3, CD14, CD16 and CD20 on the phycoerythrin (PE) channel and HLA-DR on the fluorescein-isothiocyanate (FITC) channel. DC stain positively for

HLA-DR but are negative for the cell phenotype indicators used on the PE channel (Fig. 1B). Immunoglobulin G2a (IgG2a) was used, on both the FITC and PE channel, as the isotype control (Fig. 1A).

Figures 2A and 2B show the growth kinetics of T-lymphocytes (T-cells) stimulated with autologous DC that had been pulsed with human T cell leukemia virus I (HTLV-1) tax 11-19 peptide (SEQ ID NO:1). The T-cells were subsequently restimulated weekly with HTLV-1 peptide-pulsed autologous monocytes to generate HTLV-1 peptide specific $CD8^+$ cytotoxic T-lymphocytes (CTL). An inoculum of 14.0×10^6 cells containing 22.8% $CD8^+$ T-cells was expanded to 145.0×10^6 cells containing 70.7% $CD8^+$ T-cells following 41 days of culture (Fig. 2A). The growth kinetics from a similar experiment are shown in Fig. 2B.

Figures 3A and 3B show antigen-specific lysis, measured in standard 4 hour ^{51}Cr release assay, of target (JY) cells that had been pulsed with the HTLV-1 peptide, as well as unpulsed control JY cells, by cultured T-lymphocytes that had been activated by exposure to HTLV-1 peptide-pulsed DC. Fig. 3A shows lysis measured on day 34 of the T-cell culture; Fig. 3B shows lysis measured on day 41 of T-cell culture.

Figures 3C and 3D show antigen-specific lysis, measured in standard 4 hour ^{51}Cr release assay, by cultured T-lymphocytes measured against JY (Fig. 4A) and T2 (Fig. 4B) target cells that had been either (i) unpulsed, (ii) pulsed with the HTLV-1 peptide (SEQ ID NO:1), or (ii) pulsed with the HTLV-1 peptide (SEQ ID NO:1), and then exposed to an antibody (W6/32) directed against an HLA class I antigen.

Figure 4 shows lysis of target cells endogenously expressing HTLV-1 antigens by CTL activated against HTLV-1 peptide with HTLV-1 peptide-pulsed DC.

Figure 5 are FACS profiles (filled histograms) for T-lymphocytes on day 41 of culture obtained using the indicated markers. A line histogram for the isotype control for each cell surface marker is shown superimposed on the FACS profiles.

Figures 6A and 6B are FACS profiles of dual staining with CD8 and HLA-DR antibodies, illustrating that most of the $CD8^+$ T-lymphocytes express the activation marker HLA-DR (Fig, 6B). IgG1 (PE channel) and IgG2a (FITC channel) were used as the isotype controls (Fig. 6A).

Figure 7 shows antigen-specific lysis, measured in standard 4 hour ^{51}Cr release assay, of target (JY) cells that had been pulsed with the human immunodeficiency virus (HIV) reverse transcriptase (RT) Pol 464-472 peptide (SEQ ID NO:3), as well as unpulsed

control JY cells, by cultured T-lymphocytes that had been activated by exposure to HIV peptide-pulsed DC.

Figures 8A, 8B and 8C show antigen-specific lysis, measured in standard 4 hour ^{51}Cr release assay, of target (JY) cells that had been pulsed with the HIV RT Pol 464-472 peptide (SEQ ID NO:3), as well as unpulsed control JY cells, by cultured T-lymphocytes that had been activated by exposure to HTLV-1 tax peptide-pulsed DC embedded in a collagen gel.

Figures 9A and 9B are schematic summaries of two embodiments of the PAP cell isolation method of the invention.

Figure 10 is a schematic of one embodiment of CTL expansion in response to activation by PAP cells isolated using methods of the present invention.

Figures 11A and 11B are plots of peptide-specific lysis as a function of effector to target ratio for autologous (Fig. 11A) and allogenic (Fig. 11B) CTL from HLA class I matched donor pairs.

Figures 12A and 12B are plots of peptide-specific lysis as a function of effector to target ratio for autologous (Fig. 11A) and allogenic (Fig. 11B) CTL from single allele matched donor pairs.

Figures 13A, 13B, 13C and 13D are plots of peptide-specific lysis as a function of effector to target ratio for autologous (Figs. 13A and 13C) and allogenic (Figs. 13B and 13D) CTL from single allele matched donor pairs under conditions favoring outgrowth of $CD8^+$ lymphocytes over $CD4^+$ lymphocytes.

Figure 14 shows a FACS analysis of a PAP cell fraction from a GM-CSF mobilized breast cancer donor isolated according to the methods of the present invention.

Figure 15 shows a FACS analysis of a PAP cell fraction from a healthy G-CSF mobilized donor isolated according to the methods of the present invention.

Figure 16 is a plot of the number and purity of DC before and after enrichment with mAb-coated beads.

Figures 17A, 17B and 17C are plots of T-cell proliferation as a function of effector to APC (PAP cell) ratio for control (Fig. 17A), INF peptide-pulsed (Fig. 17B) and keyhole limpet hemocyanin (KLH)-pulsed (Fig. 17C) PAP cells.

Figure 18 is a plot of the mean T-cell proliferation for control, INF peptide, inactivated influenza virus (IIV) and influenza matrix protein (IMP) in the presence and absence of W6/32 antibody.

Figures 19A and 19B are plots of peptide-specific lysis of JY cells by T-cells stimulated with either antigen alone (Fig. 19A) or antigen-pulsed DC (Fig. 19B) for control, INF peptide, IIV and IMP in the presence and absence of W6/32 antibody.

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, the terms below have the following meanings:

"Dendritic-precursor cells", or "DPC", are peripheral blood cells which can mature into dendritic cells under suitable conditions. DPC typically have a non-dendritic morphology and are not competent to elicit a primary immune response as antigen presenting cells.

"Dendritic cells", or "DC" are matured DPC, which are negative for expression of CD3, CD4, CD8, CD14, CD16 and CD20, positive for expression of HLA-DR (*i.e.*, class II MHC). Dendritic cells typically have a dendritic cell morphology -- that is, they are large veiled cells which extend dendrites when cultured *in vitro*.

"Potent antigen presenting (PAP) cells" are dendritic cells having the following characteristics: (i) after being pulsed with an antigen, PAP cells can activate naive $CD8^+$ cytotoxic T-lymphocytes (CTL) in a primary immune response, and (ii) PAP cells can be loaded with a large soluble polypeptide antigen in absence of an osmotic gradient, and can process such an antigen for presentation to MHC class I restricted $CD8^+$ CTL.

"Immunogen" refers to a substance that is able to stimulate or induce a humoral antibody and/or cell-mediated immune response.

"Antigen" refers to a substance that reacts with the products of an immune response (*e.g.*, antibodies, T-cell receptors) stimulated by a specific immunogen. Antigens therefore include the specific immunogens giving rise to the response (*e.g.*, antigenic peptides, proteins or polyssaccharides) as well as the entities containing or expressing the specific immunogens (*e.g.*, viruses, bacteria, etc.).

"Pathogen antigen" refers to an antigen derived from a microorganism having the capacity to induce disease or a diseased condition in a host organism, *e.g.*, a human. Examples of such microorganisms include viruses, bacteria, parasites and fungi.

"Tumor antigens" refer to tumor-associated antigens and tumor-specific antigens. Examples of tumor antigens include p53, carcinoembryonic antigen (CEA), HER2, MART-1 and p21RAS.

"Tumor-associated antigens" refer to antigens linked to certain tumors, such as lymphomas, carcinomas, sarcomas, and melanomas. Examples include CA-125, CA-19-9 and CA195. Tumor-associated antigens include antigens (i) absent from normal (untransformed) cells and very specific for a certain neoplasm, (ii) found on related neoplasms from separate individuals, and (iii) found on normal as well as neoplastic cells, but upregulated on the neoplastic cells relative to normal cells. Tumor-associated antigens may also be "tissue-specific" antigens, in that they are only present on or in malignant cells derived from a specific tissue. Examples of prostate tissue-specific antigens include prostatic acid phosphatase, prostate specific membrane antigen, and prostate specific antigen.

"Tumor-specific antigens" refer to antigens present on tumor cells but not found on normal cells.

II. Overview of Invention

The present invention relates to the isolation, enrichment, culture and immunotherapeutic and immunoprophylactic applications of potent antigen presenting (PAP) cells, a class of dendritic cells (DC) capable of eliciting primary immune responses in naive $CD8^+$ cytotoxic T-lymphocytes, *i.e.*, inducing naive CTL to differentiate into antigen-specific CTL. Antigen-specific $CD8^+$ CTL are the most powerful anti-tumor and anti-viral component of the immune system and have been implicated in eradication of tumors and viral infections (Grabbe, *et al.*, Guery, *et al.*, Borrow, *et al.*). Specifically, infusion of *ex vivo* activated and expanded antigen-specific $CD8^+$ CTL has been demonstrated to have therapeutic benefit in cytomegalovirus (CMV) infection and in lymphoproliferative disease (Mayordomo, *et al.*, Rooney, *et al.*, Walter, *et al.*).

DC are a morphologically similar cell type found in a number of tissues (Steinman, 1991). Three classes of DC are though to exist, but it is not known if they all derive from the same precursor or have the same function. For example, it is not known if all DC can activate naive $CD8^+$ CTL in the absence of detectable $CD4^+$ T-cells. The present invention provides, in one aspect, a method of purifying the class of DC (*i.e.*, PAP cells) that can activate naive $CD8^+$ cytotoxic T-lymphocytes (CTL) in a primary immune response after being pulsed with antigen.

According to the present invention, PAP cells are preferably obtained from peripheral blood using multiple density gradients generated from a single density gradient material. The isolation procedure can be completed in two days and can be performed

entirely under serum-free conditions. Prior to the present invention, purification of DC employed multiple density gradient materials in the separation steps and/or serum in the separation/culturing steps. The ability to perform the cell purification in the absence of serum is highly advantageous, since serum typically contains xenogeneic antigens which may be loaded by the PAP cells during purification and presented to naive T-cells, leading to T-cell activation that is not specific for the selected antigen.

The percent of PAP cells in enriched, isolated fractions may be further increased by depleting contaminating cells using, for example, solid-phase antibody-based negative depletion. Further, the isolation, enrichment and culture procedures described herein may be conveniently performed in a closed device/kit configuration.

The PAP cells present in enriched fractions are capable of eliciting both primary and secondary immune responses when co-cultured with human lymphocytes *in vitro*, and may be used in a number of applications. For example, they may be used to generate antigen-specific cytotoxic T-lymphocytes (CTL) having activity directed against major histocompatibility complex class I (MHC-I) restricted peptides from tumor- and pathogen-(*e.g.*, virus) specific antigens, for use as immunotherapy compositions.

Another aspect of the invention is directed to maintenance of the differentiated function of PAP cells by culturing them in a three-dimensional, porous, biocompatible matrix. According to the teachings herein, culturing the cells in such a matrix reduces or eliminates the need for exogenous cytokine supplementation and is effective to preserve the differentiated state of the cells for at least 12 days. Such compositions may be used, for example, to immunize individuals against selected antigens.

III. Cell Fraction Enriched for PAP Cells

A. Preparation

According to the methods of the present invention, a fraction enriched in PAP cells may be obtained by (i) obtaining, from a human blood sample, a cell fraction, preferably a monocyte-depleted cell fraction, containing peripheral blood lymphocytes and dendritic-precursor cells, (ii) culturing the cell fraction in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the morphology of dendritic cells, (iii) harvesting non-adherent cells produced by the culturing, and (iv) enriching the portion of dendritic cells in the harvested cells by density centrifugation, to obtain a fraction enriched in PAP cells.

Although the exemplified method achieves step (i) by density centrifugation, as detailed below, it will be understood that other approaches may be used to obtain such a monocyte-depleted cell fraction. For example, counterflow elutriation centrifugation (Kabel, *et al.*) may be employed to remove the monocytes.

In one general embodiment, the fraction enriched in PAP cells is obtained as detailed in Example 1, below. This procedure is based on a combination of density-based separation of cell types and differentiation-induced changes in densities of cell types following *in vitro* or *ex vivo* culture. A DPC-containing sample, such as a sample from human peripheral blood (*e.g*, buffy coats) is diluted with a suitable buffer, such as Ca^{++}/Mg^{++} free phosphate buffered saline (D-PBS; Gibco/BRL Life Technologies, Grand Island, NY), layered onto a density gradient material or separation medium (preferably having a density of about 1.0770 ± 0.0010 and an osmolarity of about 310 ± 15) and centrifuged. Exemplary density gradient materials for this step include the silica-based FEP (Ficoll Equivalent Percoll; described in Materials and Methods, below), made from "PERCOLL" (Pharmacia LKB, Uppsala, Sweden), and "LYMPHOPREP" (Nycomed Laboratories, Oslo, Norway). The separations can be carried out in any suitable tube, such as an ordinary 50 mL centrifugation tube.

The peripheral blood mononuclear cells (PBMC) at the solution interface is harvested, *e.g*, by pipeting the cells from the interface. The PBMC are then resuspended in a suitable buffer, such as D-PBS, and centrifuged to remove platelets (which remain in the supernatants).

Platelet-depleted PBMC may be either used directly in the culturing step, described below, or they may be treated to deplete monocytes, *e.g*, by an intermediate density centrifugation step. The protocol whereby the platelet-depleted PBMC are used directly in the culturing step is termed the "2-gradient" protocol, whereas the protocol where they are subjected to treatment to deplete monocytes is termed the "3-gradient" protocol.

In the 3-gradient protocol, the platelet-depleted PBMC are again resuspended in a suitable buffer, such as D-PBS, layered on a density gradient material or separation medium (preferably having a density of about 1.0650 ± 0.0010 and an osmolarity of about 300 ± 15) and centrifuged. An exemplary density gradient material for this step is the silica-based MDP (Monocyte Depletion Percoll), also made from "PERCOLL" as described below. The cells at the interface of the two solutions are primarily monocytes, while those in the pellet are primarily lymphocytes. The monocyte (interface) fraction may be resuspended in a suitable culture medium, such as cold pooled human AB serum to which an equal volume of

80% AB serum 20% dimethyl sulfoxide (DMSO) is added dropwise, and frozen until needed. The pellet cells comprise a monocyte-depleted cell fraction containing peripheral blood lymphocytes and dendritic-precursor cells (DPC).

The platelet-depleted PBMC (2-gradient protocol) or the peripheral blood lymphocytes and dendritic-precursor cells from the monocyte-depleted cell fraction (3-gradient protocol) are harvested, washed (*e.g.*, with D-PBS by centrifugation at room temperature), resuspended in a suitable culture medium, inoculated into tissue culture flasks and cultured in a humidified incubator for at least 24 hours, preferably about 40 hours. Tissue culture flasks with either adherent or non-adherent surfaces can be used. The culturing period is sufficiently-long to produce a morphological change in the dendritic-precursor cells (DPC) to cells having the morphology and characteristics of dendritic cells (DC).

This morphological change may be detected using, for example, photomicroscopy. DC are large sized veiled cells which, when cultured *in vitro*, typically extend cytoplasmic processes from the cell surface. A practical consequence of this morphological change (*i.e.*, an indicator that it has occurred) is a slight change in the cells' density, such that they become less dense. As a result of this change in density, the DC can be isolated, for example, from the interface following density centrifugation using a density gradient material or separation medium having a density of about 1.0800 ± 0.0010 and an osmolarity of about 540 ± 25, or alternatively, a density of about 1.0550 ± 0.0010 and an osmolarity of about 290 ± 15, as described below.

According to the methods of the present invention, the culture medium used in the DC isolation procedure, and particularly in the culturing step described in the above paragraph, is preferably serum-free. Experiments performed in support of the present invention and detailed in Example 1 and Table 2 herein demonstrate that the use of serum-free media in the isolation procedure and in the culturing step results in a superior purity of DC obtained in the final DC (and PAP) -enriched cell fraction.

Serum-free media which resulted in improved purity of subsequently-harvested DC included DMEM/F-12, Enriched Monocyte SFM, AIM-V and Enriched AIM-V. All of these are available from Gibco/BRL Life Technologies, Gaithersburg, MD. Other serum-free media may also be employed in the practice of the present invention. Examples include Hybridoma Serum-Free Medium (Gibco/BRL), Protein-Free Hybridoma Medium (Gibco/BRL), Iscove's Modified Dulbecco's Medium (IMDM; Sigma), X-VIVO Medium (BioWhittaker, Walkersville, MD) and MCBD medium (Sigma).

Following the culture period, non-adherent cells are harvested, for example, by gentle pipeting of the medium to dislodge cells that had settled but had not firmly adhered to the culture flask. The harvested cells are washed and resuspended either in a physiological saline solution, such as D-PBS, or in a suitable culture medium, such as one of the serum-free culture media described above. The resulting cell suspension is enriched by layering onto a density gradient material or separation medium (preferably having a density of about 1.0800 ± 0.0010 and an osmolarity of about 540 ± 25) and centrifuged. Exemplary density gradient materials for this step include the silica-based MEP, made from "PERCOLL", as well as ~14.5% Metrizamide or Nycodenz.

MEP is a hyperosmotic medium. A similar separation may be achieved using an isosmotic medium, with the density empirically adjusted downward to result in similar separation characteristics. The density is lower in the isosmotic medium because cells in isosmotic media do not lose water and shrink (*i.e.*, become more dense) the same way they do in hyperosmotic media.

An exemplary isosmotic density gradient formulation, or separation medium, useful for obtaining a PAP cell-enriched fraction is IOMEP (Iso-Osmolar Metrizamide Equivalent Percoll). IOMEP has a density of 1.0550 ± 0.0010 g/mL, an osmolarity of 290 ± 15 mOsm and a pH of 7.4 ± 0.2. It is functionally equivalent to MEP, in that it results in similar isolation yields and purities for PAP cells as are obtained on using MEP. Moreover, the function of PAP cells in the interface cell fraction, measured in terms of generation of an immune response, is similar irrespective of whether the cells were isolated on MEP or IOMEP.

Accordingly, isolation of PAP cells is dependent on the separation characteristics of the density gradient used, which in turn depends on the physical attributes such as the density, osmolarity and pH of the gradient material used. It will be appreciated that any separation medium having a combination of these characteristics such as presented above is effective for obtaining a cell fraction enriched for PAP cells.

The fraction present at the interface following the above centrifugation is enriched in DC and PAP cells. The purity of DC in this fraction may be quantified using, for example, fluorescence-activated cell sorting (FACS) analyses. DC, including the DC purified by the methods of the present invention, are typically negative for cell phenotype markers CD3 (T-cells), CD14 (monocytes), CD16 (natural killer (NK) cells) and CD20 (B-cells) and positive for HLA class II expression, as evidenced by positive staining for

HLA-DR (Macatonia, *et al.*, 1991; Markowicz and Engleman, 1990; Young and Steinman, 1987).

Figure 1 shows a representative FACS profile of the DC enriched fraction obtained using the 3-gradient protocol of the present invention. The DC purity in this profile is approximately 16.0%. On average, $0.9\pm0.7 \times 10^6$ (mean±sd) DC with a purity of $15.4\pm10.1\%$ (mean±sd) were obtained from one unit of blood (n=60). The contaminating cells following the 3-gradient protocol were mainly T-cells, B-cells, monocytes and NK-cells, in order of magnitude of contamination. The contaminating cells following the 2-gradient protocol were mainly monocytes, T-cells, B-cells and NK-cells, in order of magnitude of contamination.

If additional purification is desired, the DC and PAP-enriched fraction may be subjected to additional purification steps. For example, antibodies directed against antigens not expressed on DC, such as CD3, CD14, CD16, and/or CD20, may be immobilized on a solid support and used to remove, or "negatively deplete", contaminating cells. Such additional purification can result in further enrichment of DC, such that the DC constitute over 50% of the cells in the fraction, without appreciable loss of PAP cells.

Negative depletion experiments performed in support of the present invention (*e.g.*, Example 11; Fig. 16) demonstrated that incubation of the MEP interface fraction with beads conjugated with monoclonal antibodies for the cell surface phenotype markers CD4, CD8 and CD14 resulted in an additional ~3-fold purification (*i.e.*, ~50% purity of PAP cells obtained using the 3-gradient protocol) without appreciable loss of cell yield. Protocols for the conjugation of antibodies to beads, and for the use of such conjugated beads for negative depletions, are well known (*e.g.*, Pope, *et al.*).

The degree of enrichment of PAP cells in the final fraction may be determined using, for example, limiting dilution analysis in a CTL-activating assay. The PAP-enriched fraction is pulsed with an antigen (*e.g.*, as in the Materials and Methods), and serial dilutions of the pulsed fraction are made. The dilutions are then used to stimulate expansion of T-cells, *e.g.*, as detailed in Example 2. The relative number of PAP cells expressing the antigen in association with an MHC and capable of activating T-cells can be estimated based on the most diluted sample that results in T-cell expansion.

According to the present invention, PAP cells, which constitute a portion or all of the DC isolated by the methods of the present invention, are effective to result in the generation of a primary immune response mediated by CTL, after the PAP cells are pulsed with an antigen.

The yield and purity of PAP cells in the final fraction was evaluated as a function of the number of cells introduced into the monocyte-depleted cell fraction culture (inoculum cell concentration), as well as medium and serum composition. Results of experiments performed in support of the present invention indicate that optimal PAP cell yield and purity may be obtained if the culture is inoculated with about 2.0 - 10.0 $\times$ 10^6 MDP pellet cells per mL of culture medium.

In addition to the above serum supplemented medium, a variety of serum-free media were evaluated for DC yield and purity as described above and in Example 1, below. Results of these experiments suggested that the addition of serum had detrimental effects on both the yield and purity of PAP cells. In two of the serum-free media analyzed, AIM-V and Macrophage serum free medium (Macrophage-SFM), the yield and purity of PAP cells in the MEP interface was approximately two fold higher. These results indicate that the entire procedure of isolation and enrichment of PAP cells can be performed under serum-free conditions. This discovery is in contrast to earlier literature reports which teach that serum is an absolute requirement for *in vitro* maturation of dendritic cells (Young and Steinman, 1987; Thomas and Lipsky, 1994; Markowicz and Engleman, 1990; Mehta-Damani, *et al.*, 1994). As discussed above, serum-free purification is advantageous for isolating PAP cells that have not been inadvertently pulsed with or loaded by unselected antigens that may be present in serum. Further, the use of defined (serum-free) media in the cell purification has significant advantages from reproducibility as well as regulatory and product-development perspectives.

In order to optimize the yield of PAP cells, the percentage of PAP progenitor cells which were lost to the MDP interface following the 3-gradient protocol was quantitatively assessed. The total yield of PAP cells was calculated based on the evaluation of total cell number in the final (*e.g.*, MEP) interface fraction and the purity of PAP cells in that fraction. The yields and purity of PAP cells isolated with and without separating the PBMC on the MDP gradient were compared. The results indicate that approximately 30% of PAP cell progenitors are lost in the MDP interface fraction. Thus, to increase the total yield of PAP cells, the results presented herein indicate that the 2-gradient protocol should be employed. However, although the yield of DC was approximately 30% greater using the 2-gradient protocol, the purity of PAP cells was reduced by nearly an order of magnitude. The apparent reason for the reduction in the PAP cell purity is that following the 40 hour culture, all the cells which normally would otherwise have been separated in the MDP

interface (which is approximately one-fifth to one-third of the total number of PBMC) co-purify with the PAP cells in the MEP interface.

As illustrated in Example 10, the yield and purity of PAP cells isolated from blood of cancer patients are similar to those of PAP cells from healthy donors.

B. Characterization of Enriched Cell Fraction

DC in the enriched cell fraction typically have a dendritic morphology when cultured *in vitro*. Further, as described above, the cells are typically negative for cell surface markers CD3, CD4, CD8, CD14, CD16, and CD20, and positive for MHC class II, as evidenced, for example, by HLA-DR expression.

PAP cells in the fraction have the characteristics of the DC stated above, as well as the ability to stimulate a primary immune response mediated by MHC class I restricted CTL in response to peptides or to larger soluble antigens. The functional competence for peptides was assessed by measuring proliferative response in an allogeneic T-cell stimulation setting as detected by tritiated thymidine incorporation and by generation of peptide-specific CTL (Examples 2 and 3).

PAP cells can also be loaded with a large soluble polypeptide antigen in absence of an osmotic gradient, and can process such an antigen for presentation to MHC class I restricted $CD8^+$ CTL. Polypeptide large soluble antigens contain at least about 20 amino acid residues; preferably at least about 30 amino acid residues. Exemplary large soluble antigens include inactivated influenza virus and influenza matrix protein (IMP). Activation of CTL using PAP cells loaded with such large soluble antigens is described in Example 12.

It had been demonstrated that, in addition to peptides, certain proteins may be introduced to PAP cells such that the proteins are processed through both the MHC class I and class II pathways, as opposed to the class II pathway alone (see, for example, Mehta-Damani, *et al.*). However, prior to the present invention, loading of DC with large soluble antigenic molecules or proteins (*e.g.*, KLH) in a manner that enables presentation of the antigens to $CD8^+$ CTL has been accomplished using either incorporation of antigens into liposomes (*e.g.*, Nair, *et al.*, Reddy, *et al.*, Zhou, *et al.*) or a sucrose-induced osmotic gradient. Such an osmotic gradient causes the antigenic molecules to be taken up by the cell in vesicles resembling the types of vesicles used by the cell in processing "endogenous" antigens, and results in the presentation of the degraded antigens in association with MHC class I. An undesirable side effect of the osmotic gradient approach is a poor yield (typically $<10\%$) of viable DC or PAP cells following the loading.

C. Purified PAP Cells

According to the present invention, PAP cells purified as above constitute an exemplary APC. APC process and present antigens in association with either an MHC-I or MHC-II to T-cells. The traditional view was that "exogenous" peptides are taken up, processed (*i.e.*, degraded), and presented in association with an MHC-II to class II restricted (*i.e.*, $CD4^+$) T-cells, whereas "endogenous" peptides, such as expressed viral sequences from virus-infected cells, are presented in association with an MHC-I to class I restricted (*i.e.*, $CD8^+$) T-cells (Braciale, *et al.*, 1987). Recently, it has been shown that exogenously-added antigens can be presented to class-I restricted CTL (Moore, *et al.*, 1988).

PAP cells having the characteristics described herein, and/or isolated using the methods of the present invention may be pulsed by any of the above-described methods, or by simply incubating the antigen in the presence of PAP cells. Further, selected antigens can be introduced to PA cells by transfecting the cells with expression vectors containing genes encoding such antigens. Transfection of PAP cells with a gene encoding a desired antigen is an effective way to express the antigen in association with the class I MHC. Any of a variety of known methods (see, for example, Ausubel, *et al.*, Mulligan) may be used for such transfections, including electroporation, $CaPO_4$ precipitation, lipofection, naked DNA exposure, as well as viral vector-based approaches, such as retroviral, adenoviral, AAV, and vaccinia virus vectors.

A variety of different antigens can be loaded into and presented by PAP cells used in the practice of the present invention. For example, as described in Example 12, PAP cells can be loaded with antigenic entities ranging from small peptides to large proteins to entire viruses and subsequently used to activate T-cells, including $CD8^+$ CTL. Specific examples of antigens include infectious agents (*e.g.*, viruses, bacteria, microorganisms, and portions thereof, such as specific proteins), tumor or tumor-associated antigens expressed by cancer cells (*e.g.*, p53, carcinoembryonic antigen (CEA), HER2, MART-1 and p21RAS), and tissue-specific antigens upregulated in neoplastic transformed tissue (*e.g.*, prostatic acid phosphatase, prostate specific membrane antigen, or prostate specific antigen). Further, it will be understood that such antigens may be specifically designed and/or combined form certain desired activities. For example, a complex antigen may be recombinantly produced as a fusion of two selected antigens, as a fusion of a selected antigen and a cell targeting molecule, and the like. In a specific embodiment, such a fusion protein can include

different domains which impart (i) immunogenicity, (ii) processing via a desired MHC pathway and (iii) targeting to specific APC.

III. Biomatrix Composition

Isolated PAP cells in culture typically lose the cytoplasmic processes extending from the cell surface as well as their ability to effectively present antigens to lymphocytes for generation of primary and secondary immune responses. According to one aspect of the invention, PAP cells (pulsed or unpulsed) can be maintained in a desired (*e.g.*, active) state by culturing the cells in a three-dimensional matrix. Matrices which may be suitable for such culturing are reviewed by Langer & Bacanti. They include hydrogels, agarose (Brendel, M.D., *et al.*), and collagen (Chao, S-H., *et al.*) matrices effective to entrap the cells and provide them with a scaffold on which to grow. Such a gel or matrix is preferably stable under the conditions used for culturing. The matrix may be used to maintain a particular morphology, expression pattern or functional properties of the cells.

Experiments detailed in Example 7 describe the culturing of cells in a three-dimensional cross-linked collagen matrix. Isolated MEP interface fraction, containing PAP cells, was mixed with a solution of collagen type I monomers. This cell-collagen suspension was induced to *in situ* polymerize following a step change in pH and temperature, thus effectively entrapping the PAP in a highly porous three dimensional matrix of collagen fibers. Morphological observation of cells entrapped in collagen indicated maintenance of differentiated morphology of PAP cells following prolonged long-term culture over 12 days. Evaluation of cells released on the FACS, following digestion of the collagen matrix after 12 days of culture, indicated majority of the viable cells exhibited phenotype markers indicative of their being DC.

The entrapped PAP cells were pulsed with HLA-A*0201 binding peptide and used to generate antigen-specific $CD8^+$ T-lymphocytes. The generated CTL demonstrated peptide-specific lysis of target cells. These results indicate the ability to maintain differentiated state and antigen presentation capability of PAP cells following collagen-entrapped cultures.

These results have significant implications for the design and application of implantable or extracorporeal devices and/or systems for immuno-modulatory therapies. For example, a patient's autologous PAP cells can be isolated and entrapped in a three-dimensional system which is subsequently pulsed with antigen or peptide and used as a

vehicle for implantable or extracorporeal vaccination against native antigens to treat tumor or viral diseases.

Cell/matrix compositions of the present invention preferably contain a percentage of DC and/or PAP cells that is sufficient to stimulate a primary or secondary immune response when the composition is contacted with lymphocytes. Typically, the entrapped cells contain at least 10% DC or PAP cells, preferably at least 50% DC or PAP cells.

The PAP cells in the matrix may be modified for presentation of a selected antigen, *i.e.*, they may be "pulsed" with a peptide or protein, or transfected with a gene encoding a selected antigen. The antigen may be any antigen against which it is desired to mount an immune response, such as a tumor or viral antigen, a combination of more than one antigen (*e.g.*, a fusion protein), a combination of antigens fused to cytokines or sequences for targeting specific cell types, and the like.

IV. Utility

A. Fraction Enriched for PAP Cells

The multiple density gradients generated from a single density gradient material employed in the isolation of PAP cells may be used in a simple, closed device or kit. The PAP cells isolated using methods of the present invention may be used in a number of applications. One of the useful features of the PAP cells isolated by the methods of the present invention is that they are able to present antigens for the induction of naive and memory T-cell responses: $CD8^+$ T-cell-mediated cytotoxic responses as well as $CD4^+$ T cell-mediated proliferative responses. As such, the PAP cells are universally-useful antigen-presenting cells and can be employed in a wide range of immunotherapeutic and immunoprophylactic applications involving generation of primary and secondary immune responses.

The cells can be used, for example, in direct *in vivo* administration, *ex vivo* somatic therapy, *in vivo* implantable devices and *ex vivo* extracorporeal devices. They can also be employed in the screening of antigenicity and immunogenicity of peptide epitopes from tumor- and virus-specific antigens. PAP cells treated or pulsed with appropriate antigens can be used as potent vaccine compositions, for example against pathogenic viruses or cancerous tumors.

For *in vitro* and *ex vivo* applications, the PAP cell-containing fraction is preferably relatively pure, and contains relatively few monocytes (*e.g.*, less than about 5% monocytes). Such a fraction may be conveniently obtained using the "3-gradient" protocol

described above. For *in vivo* applications, the cell purity of the PAP cell-containing fraction is less important than the total number of PAP cells. Accordingly, a PAP fraction for *in vivo* use can be obtained using the "2-gradient" protocol described above.

The isolated PAP cells may also be used, for example, in gene therapy applications, such as transfection of the cells so that they constitutively express desired antigens/gene products for therapeutic applications.

B. Allogeneic Cells Matched for One Allele

The therapeutic benefits of antigen-specific $CD8^+$ CTL, *e.g.*, in CMV infection and lymphoproliferative disease (Mayordomo, *et al.*, Rooney, *et al.*, Walter, *et al.*) validate vaccination strategies aimed at inducing CTL responses against viruses and tumors. Recent clinical trials have yielded encouraging results wherein a cellular vaccination approach using tumor-antigen loaded autologous DC resulted in a cytotoxic T-cell immune response in melanoma (Hu, *et al.*), and tumor regression in B-cell lymphoma patients (Hsu, *et al.*).

In patients with certain advanced diseases, however, the functional potency of the DC is believed to be compromised (Borrow, *et al.*). In such cases, it may be advantageous to use cells obtained from one individual to treat a condition in a second individual. For example, the DC in breast cancer patients are functionally compromised and exhibit lower potency in stimulating T-cells compared to DC from healthy individuals (Gabrilovich, *et al.*). Similarly, multiple reports in the literature indicate that DC in AIDS patients are infected with the HIV virus (Knight, *et al.*, Macatonia, *et al.*, 1990), and that such DC are incapable of mounting a potent immune response (Macatonia, *et al.*, 1990, Chehimi, *et al.*, Eales, *et al.*).

According to the present invention, a patient's T-cells can be activated using allogenic PAP cells matched for either the entire HLA class I (Example 8) or only one HLA class I or class II allele (Example 9). This aspect of the invention thus enables a broader range of immunotherapeutic options than were previously available. For example, an AIDS patient can be vaccinated (*i.e.*, treated *in vivo*) with HIV-antigen loaded allogeneic DC from a healthy individual to generate HIV-antigen-specific $CD8^+$ CTL in the AIDS patients. Such CTL would lead to a stronger anti-viral immune response of selected specificity.

Treatment can also be carried out extracorporeally or *ex-vivo*. For example, the lymphocytes (*e.g.*, CTL) are removed from the subject and stimulated or primed with allogeneic antigen-pulsed PAP *in vitro*, and expanded. The activated lymphocytes are then injected back into the subject, where they result in the mounting of an immune response.

C. Matrix composition

The differentiated function of the PAP cells was maintained by culturing them in a three-dimensional, porous, biocompatible matrix without need for exogenous cytokine supplementation. As detailed herein (*e.g.*, Example 7), PAP cells cultured in a collagen matrix exhibited ability to elicit primary immune responses, and to maintain their differentiation state and antigen presentation capability for over 12 days. These characteristics make the cell/matrix composition useful in a number of applications.

DC or PAP cells cultured in a three-dimensional matrix may be used in application where it is desired to have a stable supply of cells in a specific state over the course of several days. For example, a three-dimensional matrix containing DC or PAP cells may be employed in implantable and/or extracorporeal devices/systems for use in immunomodulatory therapies. A patient's autologous PAP cells may be isolated and entrapped in a three-dimensional matrix such as described herein. The PAP cells, before entrapment or after entrapment in the matrix, may be pulsed with antigen or peptide and used as a vehicle for implantable or extracorporeal vaccination (*e.g.*, extracorporeal activation of lymphocytes) to treat patients with tumor or viral diseases.

Immunotherapies for individuals with defective lymph nodes are also contemplated. In such applications, a PAP cell-containing matrix is implanted into an individual to serve as an "artificial" lymph node.

An exemplary use of the cell/matrix compositions detailed herein is for immunizing a subject against a tumor or pathogen having an known tumor- or pathogen-specific antigen. In such an application, a blood-cell fraction enriched for PAP cells is isolated, *e.g.*, as described above, and the PAP cells are entrapped in a three-dimensional biocompatible matrix, such as a hydrogel, collagen gel or agarose. The cells are treated or "pulsed", either before or after the entrapping, with a selected antigen, in a manner effective to result in presentation of the antigen in association with MHC class I and class II.

If the treatment entails transforming the cells with a vector containing a gene capable of expressing the antigen, the treatment is typically done before entrapping, and transfected cells are selected (using standard methods, *e.g.*, Ausubel, *et al.*) for subsequent entrapping. Alternatively, if the treatment consists of exposing the cells to antigenic protein or peptide, the treatment may be done after the cells are entrapped.

Following the treatment of the PAP cells, the cells express the selected antigen in association with class I and class II MHC, and are thus effective to stimulate an immune response when contacted with lymphocytes (*e.g.*, a primary immune response when

contacted with naive T-cells. The treated cells entrapped in the matrix are then contacted with, or exposed to, the subject's lymphocytes. This exposure has the effect of stimulating or activating the lymphocytes such that they are capable of generating an antigen-specific proliferative and cytotoxic immune response.

In cases where the exposing is done extracorporeally, the lymphocytes (*e.g.*, CTL) are removed from the subject and exposed to the PAP cells in the matrix. The lymphocytes, many of which have been activated by the entrapped PAP cells, are injected back into the subject, where they result in the mounting of an immune response. Alternatively, the matrix containing the treated PAP cells can be injected or implanted into the subject, such that the contacting or exposing of the lymphocytes to the entrapped PAP cells occurs in the subject, and lymphocytes activated by such contacting can directly precipitate the mounting of an immune response.

The following examples illustrate, but in no way are intended to limit the present invention.

MATERIALS AND METHODS

Media

AB Culture Medium: Basal RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 2.0 mM L-Glutamine (Gibco Laboratories, Grand Island, NY) and 5% pooled human AB serum (Irvine Scientific, Santa Ana, CA).

Formulation of Density Gradients

Density gradients were prepared using "PERCOLL" (Pharmacia LKB, Uppsala, Sweden), a silica-based density gradient material (separation medium) with a density of about 1.129 ± 0.001 g/mL, an osmolarity of about 15 ± 15 mOsm and a pH of about 9.0 ± 1.0. A stock isotonic "PERCOLL" (SIP) density gradient solution was prepared by mixing the "PERCOLL" with 10X calcium/magnesium-free phosphate buffered saline (D-PBS) (Gibco Laboratories, Grand Island, NY) at a ratio of 1:9 (v/v). The following density gradient solutions were prepared by mixing the SIP solution with 1X D-PBS or 2.66X D-PBS. 2.66X D-PBS was made by mixing 10X D-PBS with and endotoxin-free (LAL) water (BioWhittaker, Walkersville, MD) in a 0.2086 ± 0.0010 to 0.8083 ± 0.0010 ratio on a weight basis. The abbreviations are as follows: FEP - Ficoll Equivalent Percoll;

MDP - Monocyte Depletion Percoll, MEP - Metrizamide Equivalent Percoll, and IOMEP - Iso-Osmolar Metrizamide Equivalent Percoll.

TABLE 1

Density Gradient Solution	Density (g/mL)	Osmolarity (mOsm/kg H_2O)	pH
SIP	1.2210 ± 0.0010	280 ± 15	7.4 ± 0.2
FEP	1.0770 ± 0.0010	310 ± 15	7.4 ± 0.2
MDP	1.0650 ± 0.0010	300 ± 15	7.4 ± 0.2
MEP	1.0800 ± 0.0010	540 ± 25	7.4 ± 0.2
IOMEP	1.0550 ± 0.0010	290 ± 15	7.4 ± 0.2

The solutions were prepared according to the following formulas, where ρ is density, v is volume, w is weight and x is the volume fraction of individual components in a mixture (m) of components (1) and (2), such that $x_1 + x_2 = 1$, $v_1 + v_2 = v_m$, and $w_1 + w_2 = w_m$. Note that the subscripts in each case refer to either component (1), component (2) or the mixture (m). Component (1) was typically SIP and component (2) was either 1X D-PBS (density of 1.0064 ± 0.0010) or 2.66X D-PBS (density of 1.0169 ± 0.0010):

$$x_1 = \frac{\rho_m - \rho_2}{\rho_1 - \rho_2} \qquad x_2 = 1\text{-}x_1$$

The formulas were entered into a spreadsheet program ("EXCEL", Microsoft Corp., Redmond, WA) to facilitate repetitive calculations of the weight of each component to generate mixtures having desired densities.

The formulated solution was characterized by measuring its (i) density on a densitometer (Model # DMA-48, Anton Paar, Ashland, VA), (ii) osmolarity on a freezing point depression osmometer (Model #2400, Fiske Instruments, Norwood, MA) and (iii) pH using a pH meter (Model #345, Corning, Corning, NY). Sterilized 1.0 M sodium hydroxide and 1.0 M hydrochloric acid solutions were used to correct the pH. Density and osmolarity were adjusted either by addition of SPI or PBS (1X, 2.66X or 10X, as required), respectively, to increase the values, or by addition of sterile water to decrease the values, to obtain a density gradient solution within the acceptable range of solution characteristics (see Table 1, above).

Elicitation and Expansion of Antigen-Specific T-Lymphocytes

Antigen-specific cytotoxic T-lymphocytes were elicited essentially as described by Mehta-Damani, *et al.*, 1994. Three HLA-A*0201 binding peptides were used to elicit antigen-specific cytotoxic T-lymphocytes (CTL). The first (SEQ ID NO:1) corresponds to amino acids 11-19 of the Tax gene product of human trophic leukemic virus 1 (HTLV-1; Elovaara, *et al.*, 1993; Kannagi, *et al.*, 1992; Zweerink, *et al.*), the second (SEQ ID NO:2) corresponds to amino acids 27-35 of the MART-1 antigen expressed on melanoma cells (Stevens, *et al.*) and the third (SEQ ID NO:3) corresponds to amino acids 464-472 of human immunodeficiency virus (HIV) reverse transcriptase in the polymerase gene. All peptides were synthesized by Bachem Laboratories (Torrance, CA).

Stock solutions were prepared by dissolving the peptides in sterile filtered 1.0% acetic acid solution in LAL water (BioWhittaker, Walkersville, MD) at a concentration of about 1 μg/ml. Isolated DC enriched cell fraction was resuspended in 1.0 mL of basal RPMI-1640 and incubated with 1 - 5 μg/mL β2-microglobulin (Sigma Chemical Company, St. Louis, MO) and 1 - 5 μg/mL peptide at 37°C for 1-2 hours. Following the incubation, peptide-pulsed DC were washed to remove excess peptide and mixed with autologous T-lymphocytes (14.5% metrizamide pellet cells) at a ratio of approximately 10:1 to yield a cell concentration of 1.0×10^6 cells/mL in AB Culture Medium supplemented with 4.0 U/mL of human recombinant IL-2 (Gibco Laboratories, Grand Island, NY). After 3 days of culture the IL-2 concentration was increased to 20.0 U/mL.

The T-lymphocytes were restimulated on a weekly schedule using autologous peptide-pulsed monocytes at a ratio of 10:1. During restimulation, the IL-2 concentration was decreased to 4.0 U/mL and was subsequently increased to 20.0 U/mL after 3 days of culture following each restimulation. CTL cultures were typically expanded for 3-4 weeks before evaluation of antigen-specific target cell lysis.

Cell-Mediated Cytotoxicity Assay

Cell-mediated cytotoxicity assays were performed using a standard 4 hour ^{51}Cr release assay. An epstein barr virus (EBV) -transfected human B-cell line, JY, and an established human T-cell line with dysfunctional transport associated protein - 1 (TAP-1) mutation resulting in presence of unoccupied HLA class I molecules on the cell surface, T2, were used as target cells for the cytotoxicity assays. Both JY and T2 cells can be obtained from the American Type culture Collection (ATCC, Rockville, MD), and were maintained in tissue culture flasks using AB Culture Medium.

The assay plates were prepared by making six serial dilutions, 1:2, of effector CTL in a final volume of 100 μL/well. Wells used for spontaneous release (background radioactive leakage) contained 100 μL/well of culture medium with no effector CTL. Wells used for measurement of maximal release (maximal ^{51}Cr incorporated into target cells) contained 50 μL culture medium and 50 μL 1.0% Triton X-100 (Sigma Chemical Company, St. Louis, MO) in LAL water.

The target cells were pulsed with peptide as described above for DC. Non-peptide-pulsed target cells and irrelevant peptide-pulsed target cells were used as controls. Target cells were washed and resuspended in 100 μL of AB serum and incubated with 100 μL of ^{51}Cr (NEN DuPont, Wilmington, DE; stock concentration = 1.0 mCi/mL) for 2 hours at 37°C. Excess unlabeled ^{51}Cr in the supernatant was washed off by three sequential centrifugal washing steps in AB Culture Medium (600 × g, 5 minutes, room temperature). Radiolabeled cells were subsequently resuspended in AB Culture Medium at a concentration of 40,000 cells/mL; 50 μL of this suspension was added to each well (2,000 cells/well) in the 96-well assay plate containing known concentrations of effector CTL.

The plates were incubated at 37°C for 4 hours in a 5% CO_2 incubator. Following the incubation, the cells were centrifuged and pelleted. One hundred μL of supernatant from each well was transferred into correspondingly-labeled wells in T-trays containing 200 μL of scintillation fluid. The T-trays were sealed and ^{51}Cr released into the supernatant was measured on a Beta-plate counter in calculated counts per minute (CCPM). The assay was set up to measure three replicate wells at each effector:target ratio for each target type. From obtained measurements, % lysis was calculated as:

$$\% \text{ Lysis} = 100 * \frac{(\text{CCPM at measured effector:target ratio} - \text{CCPM for spontaneous release})}{(\text{CCPM for maximal release} - \text{CCPM for spontaneous release})}$$

To assess whether observed lysis was HLA class I restricted, a monoclonal antibody to HLA class I, W6/32, was added to peptide-pulsed target cells at a concentration of 10-30 μg/mL to block HLA class I sites on the target cells.

Flow Cytometry

FACS analysis was done on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) connected to a Hewlett-Packard HP-9000 computer (Hewlett-Packard, Palo Alto,

CA) running "LYSIS II" software (Becton Dickinson). All monoclonal antibodies used for analysis and their respective isotype controls were purchased from Becton Dickinson. Briefly, approximately 100,000 cells were preincubated in each well of a 96-well plate with 50 μl of rabbit serum (Sigma Chemical Company, St. Louis, MO) in a final volume of 150 μL for 15-20 minutes at room temperature to block non-specific sites for antibody binding. Ten μL of desired FITC or PE -tagged monoclonal antibody were then added to the wells and the 96-well plate was incubated in the dark at 4°C for 30 minutes.

The plate was then centrifuged to pellet cells and supernatant was aspirated off to remove unbound antibody. Pelleted cells were resuspended in 100 μL of D-PBS supplemented with 5% human AB serum, fixed and counterstained by addition of 100 μL of 1.0% paraformaldehyde (Sigma Chemical Company, St. Louis, MO) supplemented with 2.0 μg/mL of LDS-751 (Molecular Probes, Eugene, OR). LDS-751 fluoresces in the far-red spectrum (PerCP region - detected by FL3 fluorescence channel on the FACScan) and counterstains cells, allowing for distinction between non-nucleated cell (non-staining), nucleated viable cell (weakly staining) and nucleated non-viable cell (very bright staining) populations.

EXAMPLE 1

ISOLATION OF DENDRITIC CELLS AND T-LYMPHOCYTES

Dendritic cells and T-lymphocytes were purified using either the "3-gradient" protocol or the "2-gradient" protocol. Unless otherwise indicated, the steps outlined below are common to both protocols. Schematic summaries of the "3-gradient" and "2-gradient" protocols are shown in Figures 9A and 9B, respectively.

Buffy coats prepared from one unit of blood from HLA-A*0201 positive volunteer healthy donors were obtained from the Stanford University Blood Center (Stanford, CA). Cells were harvested from the leukopacs, diluted to 60 mL using Ca^{++}/Mg^{++} free phosphate buffered saline (D-PBS; Gibco Laboratories, Grand Island, NY) and layered over two 15 mL columns of FEP solution, or alternatively, Lymphoprep (Nycomed Laboratories, Oslo, Norway), in 50 mL centrifuge tubes. The tubes were centrifuged at 1000 × g for 35 minutes at room temperature. The centrifuge run was allowed to stop without braking and the peripheral blood mononuclear cells (PBMC), present at the interface, were harvested.

PBMC were resuspended in D-PBS, centrifuged once at 650 × g for 10 minutes and twice more at 200 × g for 5 minutes to remove platelets.

In the "3-gradient" protocol, platelet-depleted PBMC were resuspended in 60 mL of D-PBS, layered on top of two columns of 15 mL of MDP (about 50% "PERCOLL") and centrifuged at 650 × g for 25 minutes at 4°C without braking. The MDP interface (primarily monocytes) and MDP pellet cells (primarily lymphocytes) were harvested and washed with D-PBS by centrifugation at room temperature (once at 650 × g for 10 minutes and twice thereafter at 200 × g for 5 minutes).

In the "2-gradient" protocol, the "MDP" gradient centrifugation described in the above paragraph was eliminated, and platelet-depleted PBMC were used in subsequent steps calling for either the MDP pellet fraction or the MDP interface fraction.

In instances where the PAP cells were used to generate peptide-specific CTL for purposes of elucidating their antigen presentation function, the MDP interface fraction (mostly monocytes) was resuspended in cold pooled human AB serum (Irvine Scientific, Santa Ana, CA) to which an equal volume of 80% AB serum 20% dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO) was added dropwise. The resulting cell suspension was aliquoted into cryovials and frozen in liquid nitrogen. The monocytes were used for restimulation of CTL for expansion as described in Example 2, below.

The MDP pellet fraction was resuspended in 100 mL of AB Culture Medium, inoculated into two T-75 tissue culture flasks and cultured in a humidified 5% CO_2 incubator for 40 hours. Following the incubation, the non adherent cells were harvested by moderate pipeting, washed and resuspended at a concentration of 2 - 5 × 10^6 cells/mL in AB Culture Medium.

The cell suspension was overlayered over four columns of 4.0 mL separation medium (MEP, IOMEP or ~14.5% Metrizamide (Sigma Chemical Company, St. Louis, MO)), in AB Culture Medium and centrifuged at 650 × g for 20 minutes at room temperature without braking.

The interface and pellet cells were harvested and washed in AB Culture Medium by centrifugation once at 650 × g for 10 minutes and twice thereafter at 200 × g for 5 minutes each at room temperature. The yield and viability of both cell fractions was estimated by counting on a hemocytometer using trypan blue exclusion.

The purity of DC in the interface fraction was quantified following analysis on a flow cytometer (FACS). The cells were characterized to be negative for cell phenotype markers CD3 (T-cells), CD14 (monocytes), CD16 (NK cells) and CD20 (B-cells) and positive for HLA class II expression using dual staining with HLA-DR (on the FITC channel) and a cocktail of CD3, CD14, CD16, CD20 (on the PE channel). Dual staining

with IgG2a on both the FITC and PE channels was used as isotype control. This phenotype is characteristic of DC (Macatonia, *et al.*, 1991; Markowicz and Engleman, 1990; Young and Steinman, 1987).

A representative FACS profile obtained for the DC enriched cells obtained using the 3-gradient protocol is shown in Figure 1. The DC purity in this profile is approximately 16.0%. On average, $0.9\pm0.7 \times 10^6$ (mean±sd) DC with a purity of 15.4±10.1% (mean±sd) were obtained from one unit of blood (n=60). The morphology of the cells was also evaluated using photomicroscopy. These studies indicated that the DC enriched fraction contained large sized veiled cells with cytoplasmic processes extending from the cell surface, features characteristic of DC.

The 3-gradient protocol described above was also applied in the isolation of a monocyte-depleted cell fraction containing DPC, where the fraction was cultured in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the characteristics (*e.g.*, morphological characteristics) of dendritic cells. Four different serum-free media were tested: DMEM/F-12 (Gibco/BRL Life Technologies), Enriched Macrophage SFM (Gibco/BRL Life Technologies, AIM-V (Cat #12055, Gibco/BRL Life Technologies), and Enriched AIM-V (Gibco/BRL Life Technologies). The cells were then further purified using MEP as described above, and evaluated for purity and yield of DC, as described above. The results of these experiments are presented in Table 2, below.

TABLE 2

Medium	n	Purity %	Yield %	# of DC/Buffy
RPMI + 5% AB serum	3	7.51±0.84	0.21±0.7	$0.84\pm0.28\times10^6$
DMEM:F-12 (1:1)	3	16.66±5.15	0.28±0.14	$1.12\pm0.56\times10^6$
Enriched Macrophage SFM	3	19.06±2.59	0.40±0.09	$1.60\pm0.36\times10^6$
AIM-V	2	15.50±9.50	0.35±0.28	$1.40\pm1.12\times10^6$
Enriched AIM-V	2	19.50±4.50	0.38±0.17	$1.52\pm0.68\times10^6$

The results demonstrate that the percent of DC present in fractions cultured in serum-free media (purity) is significantly greater than the purity in fractions cultured in the serum-containing control (RPMI + 5% ABS). Results of additional experiments performed in support of the present invention indicated that the addition of 50 pg/mL GM-CSF

(Granulocyte Macrophage Colony Stimulating Factor) did not increase the purity or yield of DC.

The primary difference between the PAP fractions obtained using the 2-gradient v. the 3-gradient protocols was the degree of monocyte contamination. Whereas monocytes constituted less than about 5% of cells in a PAP cell enriched fraction obtained using the 3-gradient protocol, they constituted over half of the cells obtained using the 2-gradient protocol. Accordingly, preparations isolated for use is stimulating an immune response *in vitro* or *ex vivo*, where it is preferable to reduce monocyte contamination as much as possible, were typically prepared using the 3-gradient protocol, whereas those isolated for *in vivo* or *in situ* applications may be conveniently prepared using the 2-gradient protocol.

EXAMPLE 2

GENERATION OF PEPTIDE-SPECIFIC CYTOTOXIC T-LYMPHOCYTES

The isolated cells were further confirmed to be DC by demonstrating that they retained the ability to activate naive T-cells *in vitro* using HLA class I binding peptides. DC and T-lymphocyte fractions were obtained following density gradient separation (3-gradient protocol) of peripheral blood mononuclear cells. The DC enriched fraction was pulsed with the HLA-A*0201 binding HTLV-1 peptide (SEQ ID NO:1) and cultured with autologous T-lymphocytes to generate peptide-specific CTL.

The DC enriched fraction contained 8.6×10^6 cells and the lymphocyte enriched fraction contained 139.0×10^6 cells. FACS analysis indicated DC purity of 8.0% in the DC-enriched fraction, resulting in 0.69×10^6 DC. In this instance, 2.0×10^6 interface cells (160,000 DC) were incubated with 1.0 μg/mL β2-microglobulin and 5.0 μg/mL HTLV-1 peptide (SEQ ID NO:1) for 2 hours at 37°C. The remaining DC were used to generate CTL using other HLA-A*0201 binding peptides.

The lymphocyte fraction had the following phenotype distribution when stained and analyzed on the FACS: 70.8% $CD3^+$, 49.3% $CD4^+$ and 22.8% $CD8^+$. This fraction (14.0×10^6 cells; 3.2×10^6 $CD8^+$ cells) was mixed with the HTLV-1 peptide pulsed DC-enriched cells, resulting in a DC to $CD8^+$ T-lymphocyte ratio of 1:20. Cultures were initiated at an inoculum concentration of 0.8×10^6 cells/mL in AB Culture Medium supplemented with 4.0 U/mL of IL-2. CTL were expanded by restimulating every 7 days with HTLV-1 peptide pulsed autologous monocytes and IL-2 cycling as described in the Materials and Methods for a total of 41 days, resulting in 145.0×10^6 cells containing

70.7% $CD8^+$ T-cells. A schematic summary of this expansion protocol is shown in Figure 10.

Figure 2A shows the growth kinetics of the CTL stimulated with the HTLV-1 peptide-pulsed autologous dendritic cells. Cell number initially decreases, possibly due to death of bystander cells, for approximately the first 10-12 days of culture. At day 14, during the second restimulation, the number of cells was nearly the same as the inoculated number of cells, presumably with a higher frequency of peptide-specific T-lymphocytes. Thereafter the CTL, selected by manner of antigen-specific restimulation, can be expanded beyond 40 days of culture. The specific growth rate of peptide-specific CTL from day 14 to day 41 was calculated to be 0.006 hr^{-1}, resulting in a doubling time of 4.9 days and a 45-fold expansion based on the number of inoculated $CD8^+$ cells.

Figure 2B shows the results of a similar experiment, showing the kinetics of expansion of CTL line R54, generated from 250 ml peripheral blood of a HTLV-1 seronegative individual using the HTLV-1 tax 11-19 peptide (SEQ ID NO:1). The cells expanded to over 120 $\times$ 10^6 cells after three weeks of culture, and were comprised predominantly of $CD4^+$ and $CD8^+$ T lymphocytes.

EXAMPLE 3

LYSIS OF HTLV-1 PEPTIDE-PULSED JY CELLS BY ACTIVATED CYTOTOXIC T-LYMPHOCYTES

CTL from the culture described in Example 2 were tested on day 34 and day 41 for their ability to lyse HTLV-1 peptide-pulsed JY target cells in a standard 4 hour ^{51}Cr release cytotoxicity assay (Materials and Methods). The results, shown in Figures 3A and 3B, indicated a dose response curve dependent antigen-specific lysis of peptide pulsed target cells. On day 34 (Figure 3A), at the highest effector:target ratio of 142:1, measured lysis of HTLV-1 peptide-pulsed JY cells was calculated to be 63.5$\pm$3.5 % (mean$\pm$sd) with a background lysis with unpulsed JY cells of 29.1$\pm$2.9 % (mean$\pm$sd). On day 41 (Figure 3B), at the highest effector:target ratio of 100:1 (lower than that on day 34), 82.3$\pm$1.9 % (mean$\pm$sd) and 31.8$\pm$7.5 % (mean$\pm$sd) lysis was observed for HTLV-1 peptide pulsed and unpulsed JY target cells, respectively. These results indicate that the antigen-specific cytotoxicity exhibited by generated CTL is maintained over a 40 day expansion period.

As illustrated in Figures 3C and 3D, the observed antigen-specific lysis of peptide-pulsed target cells (JY cells in Fig. 3C and T2 cells in Fig. 3D) was inhibited to levels similar to that obtained with unpulsed target cells in the presence of W6/32, a monoclonal antibody directed against HLA class I molecules. Additionally, antigen-specific

cell lysis was observed to be inhibited by the addition of OKT-8 (an antibody directed against the CD8 molecules on the T-lymphocyte surface), whereas an anti-HLA class II antibody had no effect on antigen specific lysis. These results indicate that the antigen-specific lysis is primarily HLA class I restricted and $CD8^+$ T-lymphocyte mediated.

The experiments described above were performed with HTLV-1 tax 11-19 peptide-specific CTL lines generated from the peripheral blood of 13 HLA-A2 positive, HTLV-1 seronegative individuals. Antigen-specific CTL were generated from 11 of the 13 samples.

CTL generated against HTLV-1 peptide using peptide-pulsed DC were tested for their ability to lyse target cells endogenously expressing the HTLV-1 antigens. The target cells were derived from two cell lines – MJ cells, a T-cell line obtained from the ATCC, and 20473 cells, a B-cell line obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). MJ cells are HLA-A*0201 negative ($A2.1^-$) T cells which have been infected with HTLV-1 and which synthesize HTLV-1 antigens in culture ($A2.1^-$ tax^+ T cells). A sample of MJ cells was transfected with HLA-A*0201 using transient vaccinia vector, rendering the transfected cells HLA-A*0201 positive ($A2.1^+$ tax^+ T cells). 20473 cells are an HLA-A*0201 positive transfected B-cell line obtained by insertion of a plasmid containing the Tax protein of the HTLV-1 along with a hygromycin selection gene ($A2.1^+$ tax^+ B cells). JY cells were used as the $A2.1^+$ tax^- B-cell controls.

As is illustrated in Figure 4, HTLV-1 specific CTL recognized and lysed both the HLA-A*0201-transfected MJ cells and the 20473 cells. At an effector to target ratio of 20:1 the lysis of untransfected MJ cells and transfected MJ cells was 9.72% and 39.43% respectively; and that for 20473 cells was 55.74% over a background of 6.64% with JY cells. Thus, the HTLV-1 peptide is recognized in an antigen specific, HLA-A*0201 restricted manner. These data demonstrate that the generated CTL lyse not only peptide pulsed/coated target cells, but also cells which are endogenously expressing the antigen or have been virally-infected (more analogous to an *in vivo* situation than peptide-pulsed cells).

EXAMPLE 4

PHENOTYPE ANALYSIS OF ACTIVATED T-LYMPHOCYTES

Cell phenotype analysis of the CTL described in Examples 2 and 3 was performed using FACS on day 34 and day 41 of culture. The results, shown in Figure 5, indicate that the majority of cells were either $CD4^+$ or $CD8^+$ T-lymphocytes, with no detectable numbers of NK, B or monocytic cells. Populations corresponding to cells surface markers for monocytes (CD14), NK cells (CD16) or B-cells (CD20) were not detected.

Additionally, 67.9% of the cells also expressed the activation marker HLA-DR, indicating that a significant fraction of the T-lymphocytes were activated and presumably proliferating. A line histogram for the isotype control for each cell surface marker is shown superimposed on the FACS profiles (filled histograms).

Dual staining with HLA-DR and CD8 on day 41, shown in Figs. 6A and 6B, indicated that 62.2% of the cells were both HLA-DR$^+$ and CD8$^+$, 13.8% cells were HLA-DR$^-$ but CD8$^+$ and 4.6% cells were HLA-DR$^+$ and CD8$^-$. This indicates that the majority of cells which were activated, as judged by HLA-DR expression, expressed the CD8 molecule on the cell surface, confirming that the expansion protocol led to selective expansion of antigen-specific activated CD8$^+$ T-lymphocytes.

The relative proportion of CD4$^+$, CD8$^+$ and HLA-DR$^+$ cells in culture is summarized in Table 3, below.

TABLE 3

Day	% CD4	% CD8	% HLA-DR
02	49.3	22.8	N.D.
34	43.7	58.4	60.7
41	36.4	70.7	67.9

The cell phenotype distributions were obtained following FACS staining of T-lymphocyte cultures following initiation of culture (day 2), intermediate time point (day 34) and end of culture (day 41). The results indicate an increase in the fraction of CD8$^+$ T-lymphocytes and a decrease in CD4$^+$ T-lymphocyte fraction with prolonged culture. On both day 34 and day 41, the culture is predominantly constituted of CD4$^+$ and CD8$^+$ T-lymphocytes. A majority of CD8$^+$ T-lymphocytes at the end of culture expressed the activation marker HLA-DR. These date indicate the ability to selectively expand CD8$^+$ T-cells in these cultures to generate CTL with potent peptide-specific lytic capability.

EXAMPLE 5

LYSIS OF HIV R POL 464-472 PEPTIDE-PULSED JY CELLS BY ACTIVATED CYTOTOXIC T-LYMPHOCYTES

PAP cells pulsed with the HIV RT Pol 464-472 peptide (SEQ ID NO:3) as described in the Materials and Methods were used to activate CD8$^+$ T-lymphocytes (CTL)

at a ratio of 1:10. Cultures were initiated at an inoculum concentration of 0.5×10^6 cells/mL in AB Culture Medium supplemented with 4.0 U/mL of IL-2. CTL were expanded by restimulating every 7 days with HIV Pol 464-472 peptide-pulsed autologous monocytes and IL-2 cycling as described above for a total of 21 days, resulting in 12.8 x 10^6 cells.

CTL described above were tested on day 28 for their ability to lyse HIV Pol 464-472 peptide-pulsed JY target cells in a 4 hour ^{51}Cr release cytotoxicity assay (Materials and Methods). The results, shown in Figure 7, indicated a dose response curve dependent antigen-specific lysis of peptide pulsed target cells. At the highest effector:target ratio of 10:1, measured lysis of HIV Pol 464-472 peptide-pulsed JY cells was calculated to be 57.9 ± 6.9% (mean±sd) with a background lysis with unpulsed JY cells of 26.9 ± 5.6 % (mean±sd). These results support the conclusions reached in Example 3, above, and demonstrate that antigen-specific cytotoxicity can be generated against different peptides.

EXAMPLE 6

LYSIS OF MART-1 PEPTIDE-PULSED JY CELLS BY ACTIVATED CYTOTOXIC T-LYMPHOCYTES

PAP cells were pulsed with the MART-1 peptide (SEQ ID NO:2) and used to activate and expand CTL as described above. The activated CTL were evaluated for their ability to lyse MART-1 peptide-pulsed JY target cells, unpulsed JY cells, K562 cells, Malme-3 cells (A2 negative melanoma cell line that expresses MART-1 antigen), SK-MEL-28 cells (A2 positive line that does not express MART-1 antigen) and SK-MEL-5 cells (A2 positive line that expresses MART-1 antigen).

The results show that CTL activated by MART-1 peptide-pulsed PAP were effective to lyse MART-1 peptide-pulsed JY cells, as well as the positive control SK-MEL-5 cells, at levels significantly higher than the negative control cells. Further, the results demonstrate that the CTL can lyse cells that endogenously express the MART-1 antigen.

EXAMPLE 7

THREE-DIMENSIONAL CULTURE OF DENDRITIC CELLS

Pulsed or stimulated dendritic cells were maintained in a 3-dimensional cross-linked collagen matrix for up to 12 days with no loss of ability to activate CTL. The collagen matrix was prepared by mixing three volumes of "VITROGEN-100" (2.9 mg/mL Type 1 collagen in 0.012 N HCl solution; Collagen Corp., Palo ALto, CA) with one volume of four-fold concentrated AB culture medium at 4°C, pH 7.2 (adjusted with 1.0 N NaOH).

Dendritic cells in culture were pelleted by a brief spin and were dispersed in the collagen suspension. The resulting cell-collagen mixture was then poured into multiwell plates to a final gel thickness of approximately 1 mm.

The cell/collagen mixtures were transferred to an incubator at 37°C to initiate gelation of the collagen. Gelation typically occurred in 15-20 minutes, entrapping the cells within a highly porous three dimensional network of collagen fibers. After gelation was complete, medium was added on top of the gelled plug in each well and the cultures were incubated at 37°C in a 5% CO_2 incubator.

In one series of experiments, PAP cells entrapped in collagen gel were pulsed with HTLV-1 peptide and used to stimulate autologous T-lymphocytes essentially as described above. The reagents (including the "pulsing" peptide and the T-cells) were added to the medium bathing the collagen plug containing the PAP cells. After 21 days of culture (with weekly restimulation with autologous peptide pulsed monocytes and IL-2 feeding schedule as described above), activated CTL were released from the collagen gel by digestion with collagenase.

The released cells were used in standard cytotoxicity assays as described above. Collagen entrapment culture of T-lymphocytes alone (Fig. 8B) or PAP cells (indicated as DC) alone (Fig. 8A) were used as controls. CTL generated by HTLV-1 pulsed entrapped PAP cells (indicated as DC) exhibited potent peptide specific lysis of HTLV-1 peptide pulsed target cells (JY) with low background lysis of unpulsed JY cells (Fig. 8C). The control cultures (Figs. 8A and 8B) demonstrated no appreciable antigen-specific lysis over detectable background killing.

EXAMPLE 8

ELICITATION OF PRIMARY PEPTIDE-SPECIFIC CTL RESPONSE USING ENTIRE HLA CLASS I MATCHED ALLOGENEIC PEPTIDE-LOADED PAP CELLS

PAP cell and T-lymphocyte fractions were obtained from the PBMC of HLA-A*0201 positive, HLA class I matched healthy donor pairs (Donor A and Donor B) using the 3-gradient density purification protocol described above. The HLA class I phenotype distribution for the responder (Donor A) and stimulator (Donor B) cells was A2, X, B7, B27 and A1, A2, B7, B27, respectively. The 14.5% metrizamide interface fraction yielded 0.52×10^6 PAP cells with a purity of 5.8% for the stimulators and 0.93×10^6 PAP cells with a purity of 3.3% for the responder cells.

Both PAP cell fractions were incubated with the HTLV-1 peptide as described above and used to initiate CTL cultures. Responder (Donor A) T-lymphocytes (6.25×10^6

cells) were mixed with peptide-pulsed responder (Donor A) and stimulator (Donor B) PAP cells at a ratio of 10:1 to initiate the autologous (control) and allogeneic CTL cultures, respectively. Phenotypic analysis indicated that the responder (Donor A) T-lymphocyte population was comprised of 73.3% $CD4^+$ and 11.1% $CD8^+$ T-lymphocytes. Both T-lymphocyte cultures were restimulated twice with HTLV-1 peptide-pulsed responder (Donor A) monocytes at weekly intervals.

The resulting cultures contained $8.4x10^6$ cells for the allogeneic CTL and $8.8x10^6$ cells for the autologous (control) CTL. These CTL were tested for peptide-specific lysis on day 20 of culture. The results are shown in Figures 11A and 11B. The autologous (control) CTL (Fig. 11A) demonstrated dose-responsive peptide-specific lysis of HTLV-1 peptide coated target (JY) cells. A peptide-specific lysis (filled bars) of approximately 31% was observed at an effector to target ratio of 160:1, over an undetectable background lysis, suggesting a low frequency of peptide-specific CTL in culture; not unexpected given that CTL were generated against a naive antigen.

As shown in Fig. 11B, however, CTL generated using allogeneic peptide-pulsed PAP cells demonstrated approximately 35% background lysis (open bars) at an effector to target ratio of 160:1, and exhibited a dose-response resulting in approximately 15% lysis at an effector to target ratio of 20:1. This background lysis (open bars) is likely directed against allo-antigens present on the target cells. However, a peptide-specific lysis (filled bars) was observed over the background lysis when peptide-pulsed JY cells were used as targets, with 60% lysis at an effector to target ratio of 160:1 dropping to 30% at an effector to target ratio of 20:1. These results indicate that allogeneic PAP cell are able to stimulate a primary immune response *in vitro*.

Analysis of cell phenotype of the allogeneic and autologous (control) CTL is depicted in Table 4, below.

Table 4

%	CULTURE INITIATION (DAY 2)	ASSESSMENT OF CYTOTOXICITY (DAY 20)	
	RESPONDER T-CELLS	AUTOLOGOUS CTL	ALLOGENEIC CTL
% CD4 (total)	73.3%	80.2%	67.6%
%CD4 (activated: HLA:-DR^+)		2.5%	37.9%
%CD4 (not activated: HLA:-DR^-)		77.7%	29.7%

% CD8 (total)	11.1%	16.4%	19.8%
%CD8 (activated: HLA:-DR$^+$)		6.8%	16.5%
%CD8 (not activated: HLA:-DR$^-$)		9.6%	3.3%

Both CTL lines consisted predominantly of CD4$^+$ T-lymphocytes, presumably a reflection of the high CD4$^+$ T-lymphocyte content (CD8$^+$/CD4$^+$ ratio = 0.15) in the starting T-lymphocyte population for the responder. In the autologous (control) CTL line (CD8$^+$/CD4$^+$ ratio = 0.21) 3.1% of the CD4$^+$ and 41.5% of the CD8$^+$ T-lymphocytes expressed the activation marker HLA-DR. In contrast, in the allogeneic CTL line (CD8$^+$/CD4$^+$ ratio = 0.29), 56.1% of the CD4$^+$ and 83.3% of the CD8$^+$ T-lymphocytes expressed the activation marker HLA-DR.

The presence of larger fractions of activated (HLA-DR expressing) populations of both CD4$^+$ and CD8$^+$ T-lymphocytes in the allogeneic CTL line is likely due to allo-antigen specific responses induced by the allogeneic PAP cell which caused the high background lysis. However, in both allogeneic and autologous (control) CTL lines, the ratio of the CD8$^+$ to CD4$^+$ T-lymphocytes increased from 0.15 to 0.29 and 0.21, respectively, indicating that the expansion protocols described herein lead to favorable conditions for the expansion of CD8$^+$ over CD4$^+$ T-lymphocytes.

EXAMPLE 9

ELICITATION OF PRIMARY PEPTIDE-SPECIFIC CTL RESPONSE USING SINGLE HLA CLASS I ALLELE MATCHED ALLOGENEIC PEPTIDE-LOADED PAP CELLS

Buffy coats prepared from volunteer healthy donors (of unknown HLA genotype) at Stanford University Blood Center (Stanford, CA) were typed on the FACS using a FITC-conjugated antibody (BB7.2; generated from a hybridoma, HB-82, purchased from the ATCC) specific for the HLA-A*0201 molecule. Buffy coats sharing the HLA-A*0201 allele were used as responders and stimulators in the single HLA class I allele matched experiments.

Single allele matched allogeneic PAP cells were used to generate peptide-specific CTL as described in Example 8, above. Results from a cytotoxicity assay performed on day 18 for a representative experiment are depicted in Figures 12A and 12B. As in Figs. 11A and 11B, open bars represent background lysis and filled bars represent peptide-specific lysis. It can be appreciated that no peptide-specific lysis was detected over background with the allogenic generated CTL (Fig. 12B), whereas the autologous (control)

CTL (Fig. 12A) yielded potent peptide-specific lysis. These results were reproduced in multiple experiments.

Additionally, phenotypic analysis of generated CTL product indicated that in case of the allogeneic CTL, a high proportion of the $CD4^+$ and $CD8^+$ T-lymphocytes were activated (as judged by the expression of the activation marker HLA-DR). For example, in the case of the experiment shown in Figs. 12A and 12B, 49.2% of $CD4^+$ and 42.7% of $CD8^+$ T-lymphocytes were $HLA\text{-}DR^+$ in the allogeneic CTL compared to 1.0% of $CD4^+$ and 4.2% of $CD8^+$ being activated in the autologous (control) CTL.

The results described above suggest that although allogeneic PAP cell are capable of eliciting a primary peptide-specific immune response *in vitro*, the response against allo-antigens may mask the detection of a peptide-specific response. Further, it appears that this effect may be more drastic if only one HLA class I allele is matched, rather than a complete HLA class I match between the T-cells and PAP cell.

The above-described protocols were modified in two ways to address these issues -- First, the cells were maintained in culture longer and received five instances of weekly restimulation with peptide-pulsed autologous monocytes; and second, the culture conditions were modified to provide selective growth advantage for the outgrowth of $CD8^+$ T-lymphocytes over the $CD4^+$ lymphocytes by increasing the concentration of rIL-2 from 20U/mL to between 400-1000 U/mL three days subsequent to every restimulation.

The results of two representative experiments (R38 and R40) are described herein. The 14.5% metrizamide interface fraction in experiment R38 contained $1.3x10^6$ cells with a PAP cell purity of 43.3% (resulting in $0.563x10^6$ PAP cell being obtained) while that of R40 contained $11.3x10^6$ cells with a PAP cell purity of 6.4% (resulting in $0.723x10^6$ PAP cell being obtained). The T-lymphocyte subsets consisted of 64.1% $CD4^+$ and 21.5% $CD8^+$ T-lymphocytes for R38 and 41.9% $CD4^+$ and 43.5% $CD8^+$ T-lymphocytes for R40.

Four CTL cultures were initiated using R38 and R40 PAP cell and T-lymphocytes as both responders and stimulators -- R38 autologous (control): R38 PAP cell with R38 T-lymphocytes; R38 allogeneic: R40 PAP cell with R38 T-lymphocytes; R40 autologous: R40 PAP cell with R40 T-lymphocytes; and R40 allogeneic: R38 PAP cell with R40 T-lymphocytes. The phenotype analysis on day 13 of culture indicated the following distribution between $CD4^+$ and $CD8^+$ T-lymphocytes: 82.6% $CD4^+$ and 9.7% $CD8^+$ T-lymphocytes for R38 autologous (control); 72.9% $CD4^+$ and 14.6% $CD8^+$ T-lymphocytes for R38 allogeneic; 56.3% $CD4^+$ and 9.3% $CD8^+$ T-lymphocytes for R40 autologous (control); 78.0% $CD4^+$ and 15.1% $CD8^+$ T-lymphocytes for R40 allogeneic.

Cytotoxicity assay was performed on day 13, wherein no detectable peptide-specific lysis was observed for either of the four CTL lines. Subsequent to the second restimulation on day 14 and every restimulation thereafter, the IL-2 concentration following 3 days after restimulation was increased to 400-1000 U/mL.

The CTL lines contained the following expanded cell numbers: $85.4x10^6$ cells for R38 autologous (control) CTL, $96.4x10^6$ cells for R38 allogeneic CTL, $131.8x10^6$ cells for R40 autologous (control) CTL, and $116.1x10^6$ cells for R40 allogeneic CTL. Peptide-specific lysis determined at day 34 is shown in Figures 13A and 13B (R38); and Figures 13C and 13D (R40). It can be appreciated from the Figures that all four CTL lines exhibited potent peptide-specific lysis (greater than 40% lysis at an effector to target ratio of 50:1) with background lysis being less than 10% for all four CTL lines (bars as described above with respect to Figs. 11A, 11B, 12A and 12B). Analysis of cell phenotype of the allogeneic and autologous (control) CTL as depicted in Table 5, below, indicated the following distribution between $CD4^+$ and $CD8^+$ T-lymphocytes: 71.1% $CD4^+$ and 14.8% $CD8^+$ T-lymphocytes for R38 autologous (control); 60.6% $CD4^+$ and 37.1% $CD8^+$ T-lymphocytes for R38 allogeneic; 66.0% $CD4^+$ and 31.0% $CD8^+$ T-lymphocytes for R40 autologous (control); 85.7% $CD4^+$ and 10.8% $CD8^+$ T-lymphocytes for R40 allogeneic.

Table 5

	Culture Initiation (Day 2)		Assessment of Cytotoxicity (Day 34)			
	Responder T-Cells		Autologous CTL		Allogeneic CTL	
	R38	R40	R38	R40	R38	R40
% CD4 (total)	**64.1%**	**41.9%**	**71.1%**	**66.0%**	**60.6%**	**85.7%**
%CD4 (activated: HLA:-DR^+)	2.7%	1.3%	2.5%	11.3%	7.4%	21.0%
%CD4 (not activated: HLA:-DR^-)	61.4%	40.6%	68.6%	54.7%	53.2%	64.7%
% CD8 (total)	**21.5%**	**43.5%**	**14.8%**	**31.0%**	**37.1%**	**10.8%**
%CD8 (activated: HLA:-DR^+)	1.2%	3.7%	2.4%	12.7%	11.4%	5.9%
%CD8 (not activated: HLA:-DR^-)	20.3%	39.8%	12.4%	18.3%	25.7%	4.9%

The results described above indicate that manipulation of culture conditions allows the suppression of the allo-antigen specific component of the response and the selective

expansion the peptide-specific $CD8^+$ CTL response even if only one allele is matched between the PAP cell and the T-lymphocytes.

EXAMPLE 10

Isolation of Cell Fraction Enriched in PAP Cells From Peripheral Blood of Mobilized Healthy Donors and Cancer Patients

Blood samples were obtained following systemic granulocyte colony stimulating factor (G-CSF) -mobilization of a normal healthy volunteer donor and granulocyte-macrophage colony stimulating factor (GM-CSF) -mobilization of a breast cancer patient. PAP cell fractions were isolated using the 3-gradient protocol as described in Example 1 and were stained for FACS analysis using PE conjugated monoclonal antibodies (mAb) to cell surface markers specific for T- ($CD3^+$), monocytic- ($CD14^+$), NK like- ($CD16^+$) and B- ($CD20^+$) cell lineage and a FITC conjugated mAb to the DR allele of HLA class II, an activation marker of PAP cells. The FACS profiles are shown in Figures 14 and 15 for the healthy and breast cancer donors, respectively.

The PAP cell enriched fraction (interface of MEP, IOMEP or ~14.5% Metrizamide) from the breast cancer donor contained 8.0×10^6 and 6.0×10^6 cells with 4.1% and 5.8% dendritic cells, resulting in 0.33×10^6 and 0.35×10^6 dendritic cells from non-adherent and adherent cultures, respectively (Figure 14), yielding a total of 0.68×10^6 dendritic cells from one unit (approximately 500 mL) of whole blood. The PAP cell enriched fraction from the healthy G-CSF mobilized donor contained 8.7×10^6 cells with 8.2% dendritic cells, resulting in 0.72×10^6 dendritic cells from one unit of whole blood (Figure 15). The average purity of dendritic cells in the PAP cell enriched fraction obtained from normal (unmobilized) healthy volunteer donors was $15.4 \pm 10.1\%$ (mean$\pm$sd), resulting in $0.9 \pm 0.7 \times 10^6$ (mean$\pm$sd) dendritic cells from one unit of whole blood.

These results indicate that the purity and number of dendritic cells obtained from mobilized healthy donors and mobilized cancer patients was similar to that obtained from healthy unmobilized donors.

EXAMPLE 11

Enrichment of Dendritic Cells From the PAP Cell Fraction Using Negative Depletion for Contaminating Lineage Positive Cells

PAP cell enriched fractions were obtained from peripheral blood of normal healthy volunteer donors using the 3-gradient protocol described in Example 1. The MEP interface

contained 4.9 x 10^6 cells, of which 16.3% (0.80 x 10^6) were dendritic cells. The interface cells were further split into three parts and incubated with beads coated with monoclonal antibodies (mAb) directed against cell surface markers for either T- cells ($CD4^+$ or $CD8^+$) or monocytes (CD14). The resulting suspension of cells and beads was layered on top of the FEP density gradient and centrifuged. The cells which had bound to the mAb-coated beads settled into the pellet and were discarded.

The unbound cells were harvested, enumerated and analyzed on the FACS for dendritic cell content. The analysis showed that this cell fraction was substantially enriched for dendritic cells -- of a total of about 1.68 x 10^6 cells recovered, approximately 49.5% (0.83 x 10^6) were dendritic cells. The results are plotted in Figure 16. Note that the total number of DC before and after this enrichment step remained essentially constant, illustrating that such a solid support-based method can be used to significantly enrich the preparation for dendritic cells without incurring a substantial loss of the dendritic cells.

EXAMPLE 12

LOADING OF THE PAP CELL FRACTION WITH TUMOR- OR VIRUS-SPECIFIC ANTIGENS RESULTS IN THE ELICITATION OF A PRIMARY ANTIGEN-SPECIFIC PROLIFERATIVE AND CYTOTOXIC IMMUNE RESPONSE

PAP cell enriched fractions were obtained from peripheral blood of normal healthy volunteer donors using the 3-gradient protocol described in Example 1. The pellet cell fraction of the MEP (or IOMEP or 14.5% Metrizamide) separation was used as the source of T lymphocytes either unaltered (whole T-cells) or following depletion of $CD4^+$ cells with CD4-coated magnetic beads (enriched for $CD8^+$ T-cells). These two responder population of cells (whole T-cells and $CD8^+$ T-cells) were used in subsequent assays for proliferative and cytotoxic response elicitation.

The PAP-cell enriched fraction was pulsed with antigen by incubation for 1 hour at 37°C. The antigens used were either a naive antigen (keyhole limpet hemocyanin; KLH) or a memory antigen (influenza matrix protein; IMP). A naive antigen is one which has not been previously encountered by the immune system while a memory antigen is one to which the cells in the immune system have been pre-exposed by either infection or inoculation. An HLA-A2 restricted peptide from the IMP (INF peptide) was used as a control, since other experiments performed in support of the present invention demonstrated that peptide-pulsed PAP cell fractions are capable of generating a peptide-specific $CD8^+$ cytolytic T lymphocyte (CTL) response.

In an exemplary experiment, PAP enriched cells were phenotyped on the FACS and found to contain 13.84% dendritic cells (as adjudged by being negative for lineage markers CD3, CD14, CD16, CD20 and positive for the expression of the activation marker, HLA class II DR allele; see Example 1). These cells were loaded with either no antigen, KLH or the INF peptide and mixed with responder cells (whole T-cells or $CD8^+$ T-cells) at varying ratios of antigen presenting cells to responder T-cells (1:2 through 1:128 at two-fold serial dilutions). The resulting cultures were inoculated in 96-well culture plates, at a concentration of 10^5 responder T-cells per well in 200μL of 5% human AB serum supplemented culture medium and were incubated in a 37°C, 5% CO_2 in air incubator for five days. On the fifth day, each well in the 96-well culture plate was pulsed with 0.5 μCi/well of tritiated thymidine (^{3}H-TdR). Plates were harvested on the sixth day and the amount of incorporated ^{3}H-TdR was measured on a ß-plate scintillation counter.

The results, illustrated in Figures 17A, 17B and 17C, indicate that the responder cells (both whole T-cells and CD8+ T-cells) can proliferate in response to stimulation with antigen-loaded PAP cell enriched fraction. Responder cell proliferation was detected against both memory antigen and naive antigen. Note that these antigens by themselves do not stimulate a T-cell proliferative response and hence need to be presented to the surface of the antigen presenting cells. These results further confirm that the PAP cell enriched fraction contains cells which have potent immunostimulatory capacity. Additionally, this dose-dependent, antigen-specific proliferation was detected with both whole T-cells ($CD4^+$ and $CD8^+$ T-cells) and with $CD8^+$ T-cells ($CD4^+$ depleted whole T-cells), indicating that the immune response generated on elicitation employing antigen-loaded PAP cells was mediated via both the HLA class I and the HLA class II pathways. Although it is generally believed that T-cell proliferation in response to soluble antigens (whole proteins) is primarily HLA class II mediated, the results presented herein indicate that even CD4-depleted T-cells (primarily $CD8^+$ T-cells) can proliferate in response to soluble protein antigens, if the antigens are presented by antigen presenting cells in the PAP cell enriched fraction.

These observations were supported by results of experiments similar to those described above, but which included an additional control whereby the proliferation response was measured in the presence of blocking with a HLA class I specific monoclonal antibody, W6/32. CD4-depleted T-cells ($CD8^+$ T-cells) were used as responder cells, influenza matrix protein (IMP) was used as the antigen, and IMP-loaded PAP cells were employed as stimulators at stimulator to responder ratios of 0, 1:2, 1:8 and 1:32. The

results, shown in Figure 18, indicate that the proliferative response obtained for $CD8^+$ T-cells can be blocked by the presence of W6/32, confirming the observation that the immune response generated to soluble antigens employing antigen-loaded PAP cells is mediated via both the HLA class I and the HLA class II pathways.

The immune response elicited by antigen-loaded PAP cells, as detected by proliferation of $CD4^+$ and $CD8^+$ T-cells, should result in the generation of a CD4 helper response and a CD8 cytotoxic response. The ability to use soluble antigens to generate a CD4 response is well documented in the literature. Experiments were designed to assess whether the CD8 proliferative response resulted in the establishment of antigen-specific $CD8^+$ CTL.

A PAP cell fraction obtained as described above was loaded with either the INF peptide, an influenza vaccine consisting of inactivated influenza virus, or the influenza matrix protein (IMP). PAP cells not loaded with any antigen were used as controls. The antigen-loaded PAP cells were mixed with autologous T-lymphocytes and used to initiate CTL cultures as described in Example 2. One week after stimulation with antigen-loaded PAP cells, the cultures were expanded non-specifically for one week using phytohemagglutinin (PHA) and interleukin-s (IL-2). The resulting cells were tested in a standard 4 hour chromium release cytotoxicity assay as described in the Materials and Methods and Example 3. JY cells, a B-cell line expressing the HLA-A*0201 allele, were used as target cells. Antigen-specific lysis was measured as the killing of JY cells pulsed with the INF peptide versus the background killing of unpulsed JY cells. Additionally, blocking with W6/32 was employed to confirm that the cytotoxicity was attributable to $CD8^+$ T-cells and was mediated by HLA class I interactions between the effector and target cells. The results, shown in Figures 19A and 19B, indicate potent INF peptide-specific lysis by T-cell lines stimulated with PAP cells loaded with INF peptide, inactivated influenza virus, and influenza matrix protein (IMP). Additionally, the results show that antigen-specific lysis was blocked by the monoclonal antibody W6/32, directed against HLA class I molecule, confirming that the lysis was mediated by $CD8^+$ CTL and HLA class I restricted.

Taken together, the results shown in Figures 17A-C, 18, 19A and 19B, demonstrate that both HLA class I and HLA class II mediated proliferative and cytotoxic T-cell responses can be generated to memory and naive antigens *in vitro* using antigen-loaded PAP cells.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Peshwa, Madhusudan V.
van Shooten, Willem C.

(ii) TITLE OF INVENTION: Potent Antigen Presenting Cell Method and Composition

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dehlinger & Associates
(B) STREET: 350 Cambridge Avenue, Suite 250
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94306

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: 12-JUL-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sholtz, Charles K.
(B) REGISTRATION NUMBER: 38,615
(C) REFERENCE/DOCKET NUMBER: 7636-0008

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 324-0880
(B) TELEFAX: (415) 324-0960

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Peptide HTLV-1 Tax 11-19 Pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Leu Phe Gly Tyr Pro Val Tyr Val
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Peptide MART-1 Pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Ala Gly Ile Gly Ile Lys Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Peptide HIV Pol 464-472 Pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile Leu Lys Glu Pro Val His Gly Val
1 5

IT IS CLAIMED:

1. A method for obtaining, from a human blood sample, potent antigen presenting (PAP) cells characterized by (i) a phenotype that is positive for surface antigen HLA DR and negative for surface antigens CD3, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes in culture, said method comprising

obtaining from the blood sample, a cell fraction containing peripheral blood lymphocytes and dendritic-precursor cells,

culturing the cell fraction in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the morphology of dendritic cells,

harvesting non-adherent cells produced by said culturing, and

enriching the portion of dendritic cells in the harvested cells by density centrifugation, to obtain a fraction enriched in PAP cells.

2. A method of claim 1, wherein said cell fraction, containing peripheral blood lymphocytes and dendritic-precursor cells, obtained from said blood sample, is a monocyte-depleted cell fraction.

3. A method of claim 2, wherein said obtaining includes (i) first enriching the blood sample in peripheral blood mononuclear cells by density centrifugation, and (ii) enriching the product of (i) in lymphocytes and dendritic-precursor cells by density centrifugation.

4. A method of claim 3, wherein enriching step (ii) is carried out by layering the product of (i) over a separation medium having a density of 1.0650 ± 0.0010 g/mL, and an osmolarity of 300 ± 15 mOsm.

5. A method of any of claims 1-4, wherein said culturing is carried out in an AIM-V or macrophage serum-free medium, for a period of at least 24 hours.

6. A method of claims 1 or 2, wherein said enriching is carried out by layering the harvested non-adherent cells over a separation medium having a density of 1.0800 ± 0.0010 and an osmolarity of 540 ± 25 mOsm.

7. A method of claims 1 or 2, wherein said enriching is carried out by layering the harvested non-adherent cells over a separation medium having a density of 1.0550 ± 0.0010 and an osmolarity of 290 ± 15 mOsm.

8. A method of claims 1 or 2, which further includes, following said enriching, contacting the cells in the PAP-enriched fraction with a solid phase support conjugated with antibodies against at least one cell surface phenotype marker selected from the group consisting of CD3, CD4, CD8, CD14, CD16, and CD20, and removing cells in the fraction which bind to the solid phase.

9. A method of claim 8, wherein the solid phase support is conjugated with antibodies against CD3, CD14, CD16, and CD20.

10. A method of claim 9, wherein the cells remaining after removing cells binding to the solid phase support are over 50% dendritic cells.

11. A method of claim 10, for use in preserving the differentiation state and antigen presentation capability of the PAP cells in culture for an extended culture period, which further includes entrapping cells from the PAP-enriched fraction in a three-dimensional matrix.

12. A method of claim 11, wherein the three-dimensional matrix is a crosslinked collagen matrix.

13. A cell composition comprising potent antigen presenting (PAP) cells characterized by (i) a phenotype that is positive for surface antigen HLA DR and negative for surface antigens CD3, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes in culture, entrapped in a three-dimensional matrix.

14. A composition of claim 13, wherein the three-dimensional matrix is a cross-linked collagen matrix.

15. A composition of claims 13 or 14, wherein the PAP-cells in the composition are prepared by

obtaining from a human blood sample, a monocyte-depleted cell fraction containing peripheral blood lymphocytes and dendritic-precursor cells,

culturing the cell fraction in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the characteristics of dendritic cells,

harvesting non-adherent cells produced by said culturing, and

enriching the portion of dendritic cells in the harvested cells by density centrifugation, to obtain a fraction enriched in PAP cells.

16. A composition of claim 15, wherein the entrapped cells include at least 10% PAP cells.

17. A composition of claim 15, wherein the entrapped cells include at least 50% PAP cells.

18. A composition of any of claims 13-17, wherein the PAP cells in said matrix are modified for presentation of a selected antigen.

19. A composition of claim 18, wherein the selected antigen contains a pathogen antigen or a tumor antigen.

20. A composition of claim 19, wherein the selected antigen is a prostate tissue-specific tumor antigen selected from the group consisting of prostatic acid phosphatase, prostate specific membrane antigen, and prostate specific antigen.

21. A composition of claim 19, wherein the selected antigen is a tumor antigen selected from the group consisting of p53, carcinoembryonic antigen (CEA), HER2, MART-1 and p21RAS.

22. A composition of claim 21, wherein the selected antigen is peptide MART-1 27-35 Pep (SEQ ID NO:2).

23. A composition of claim 19, wherein the selected antigen is a viral pathogen antigen selected from the group consisting of human immunodeficiency virus (HIV) pol, human T cell leukemia virus I (HTLV-1) tax and influenza matrix protein (IMP).

24. A composition of claim 23, wherein the selected antigen is peptide HIV Pol 464-472 Pep (SEQ ID NO:3) or peptide HTLV-1 Tax 11-19 Pep (SEQ ID NO:1).

25. A method of immunizing a subject against a tumor or pathogen having an known tumor- or pathogen-specific antigen, comprising

isolating from the subject, a blood-cell fraction enriched for potent antigen presenting (PAP) cells characterized by (i) a phenotype that is positive for surface antigen HLA DR and negative for surface antigens CD3, CD14, CD16, and CD20, and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes in culture,

entrapping the PAP cells in a three-dimensional biocompatible matrix,

before or after said entrapping, pulsing the matrix-entrapped PAP cells with the selected antigen, and

exposing the subject's lymphocytes to the matrix.

26. A method of claim 25, wherein said isolating includes obtaining from a human blood sample, a cell fraction containing peripheral blood lymphocytes and dendritic-precursor cells,

culturing the cell fraction in a serum-free medium for a period sufficient to produce a morphological change in dendritic-precursor cells to cells having the characteristics of dendritic cells,

harvesting non-adherent cells produced by said culturing, and

enriching the portion of dendritic cells in the harvested cells by density centrifugation, to obtain a fraction enriched in PAP cells.

27. A method of claims 25 or 26, wherein said exposing includes injecting the matrix into the subject.

28. A method of claims 25 or 26, wherein said exposing includes removing cytotoxic T lymphocytes from the subject, contacting the lymphocytes with the matrix *ex vivo*, and returning the contacted lymphocytes to the subject's bloodstream.

5

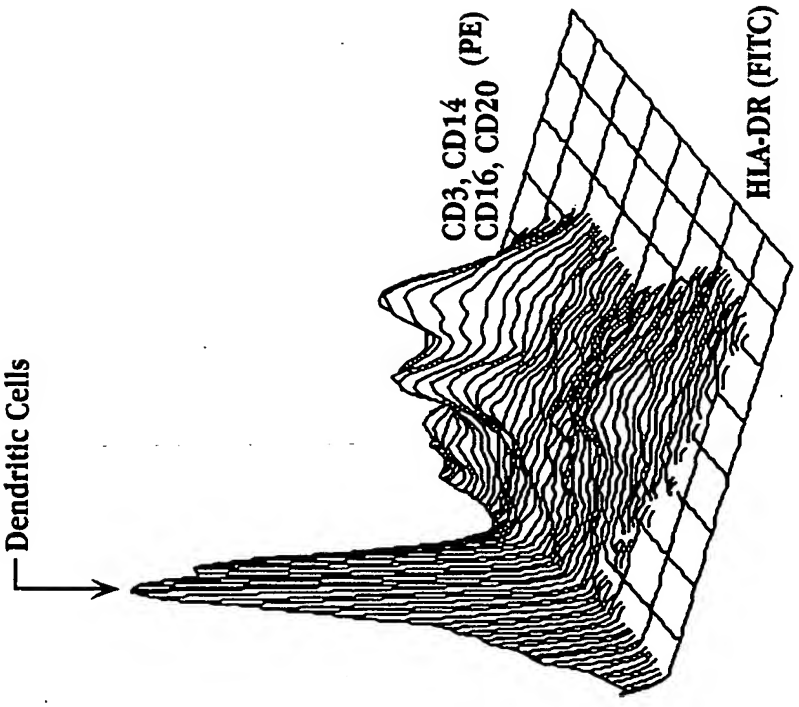

Dendritic Cells
CD3, CD14
CD16, CD20 (PE)
HLA-DR (FITC)

Fig. 1B

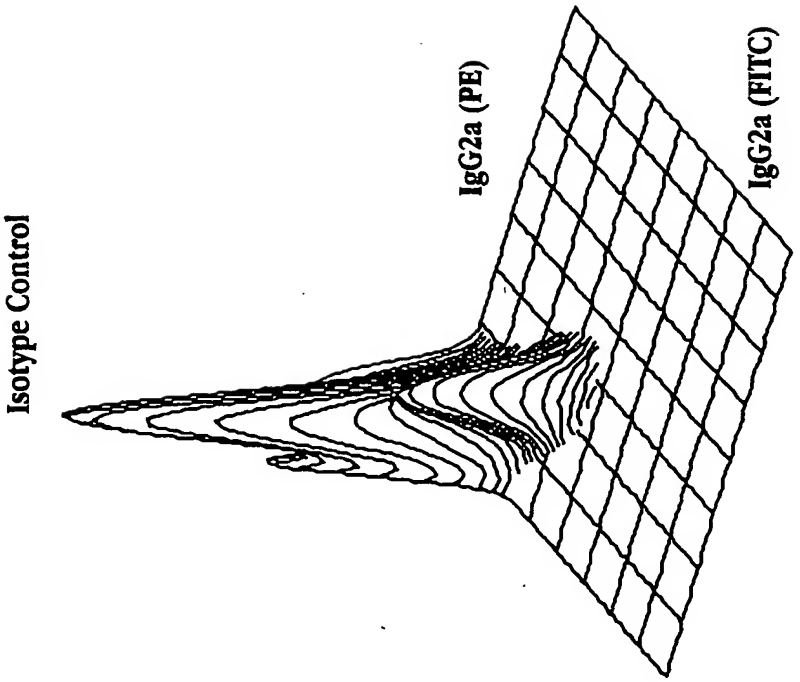

Isotype Control
IgG2a (PE)
IgG2a (FITC)

Fig. 1A

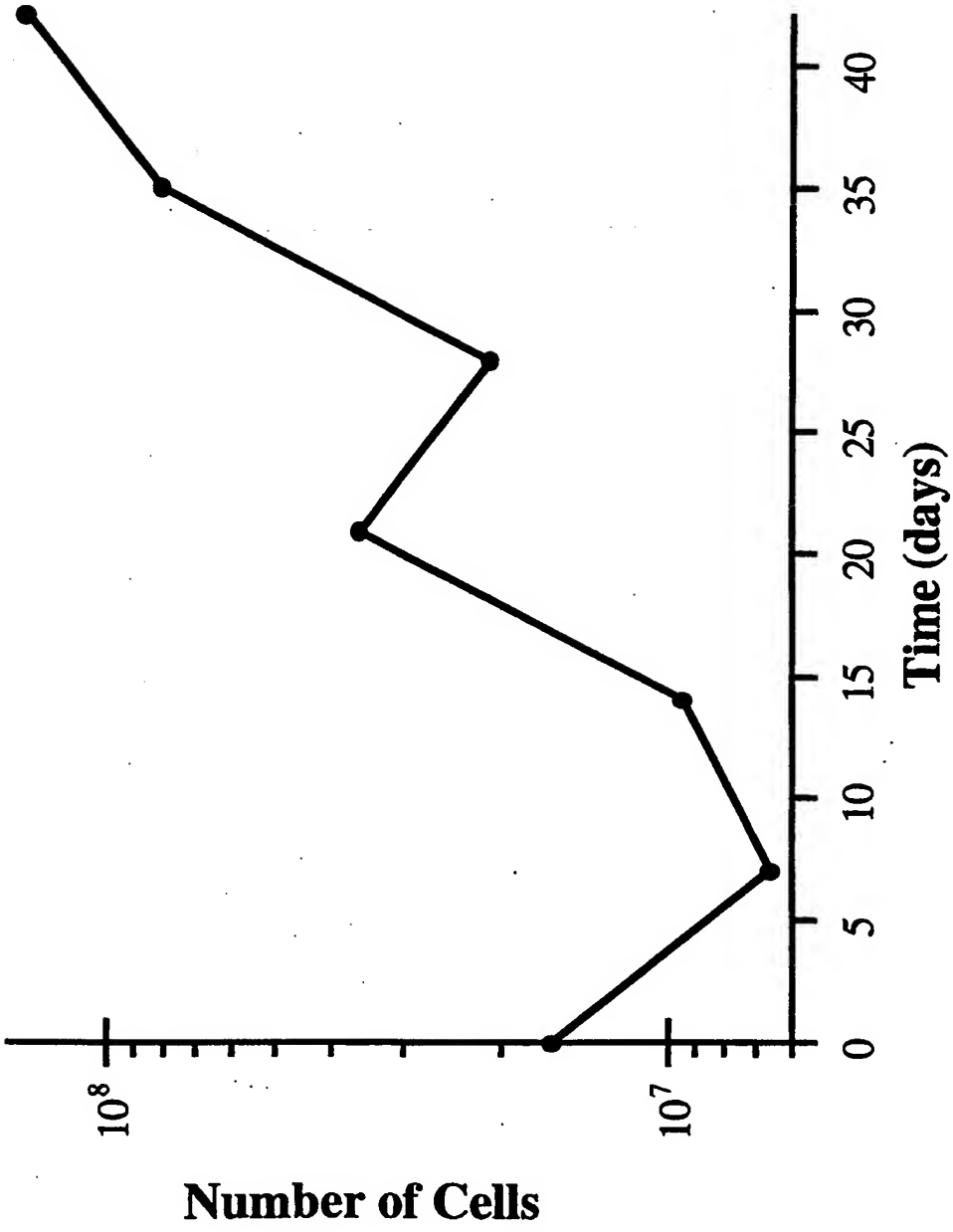


Fig. 2A

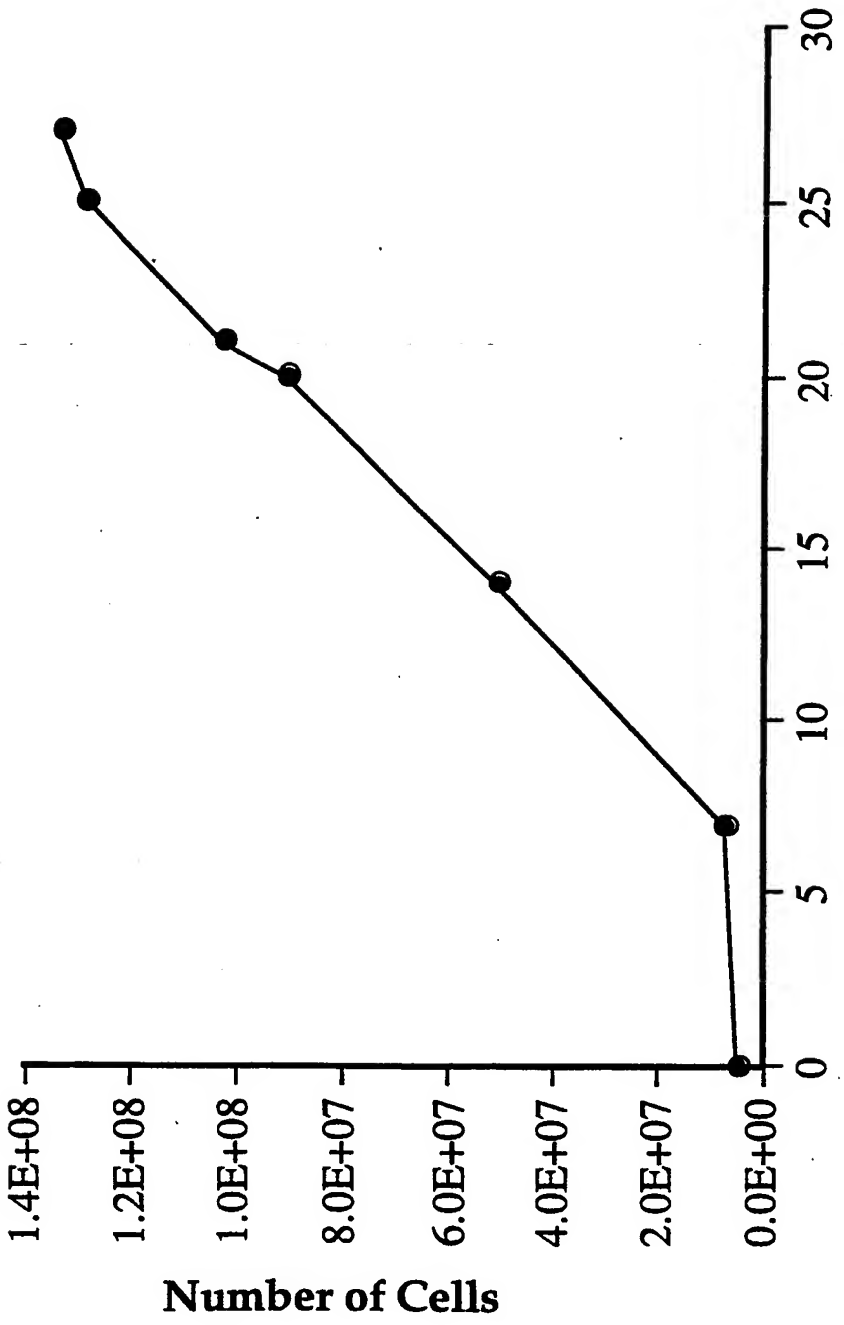

1.4E+08
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
Number of Cells
0
5
10
15
20
25
30
Time (days)

Fig. 2B

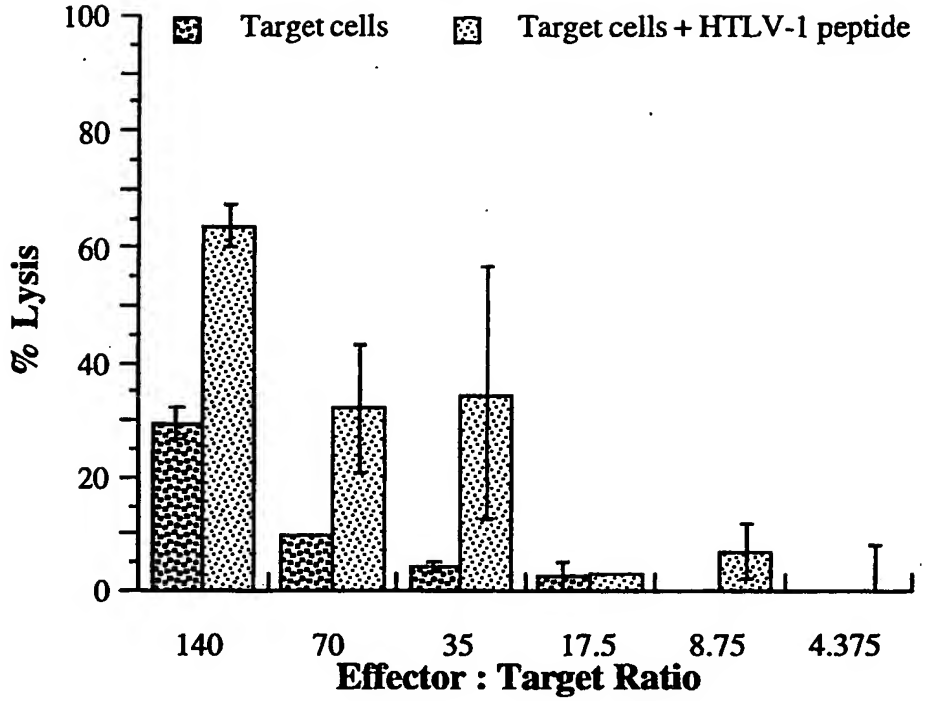

Target cells
Target cells + HTLV-1 peptide
% Lysis
100
80
60
40
20
0
140
70
35
17.5
8.75
4.375
Effector : Target Ratio

Fig. 3A

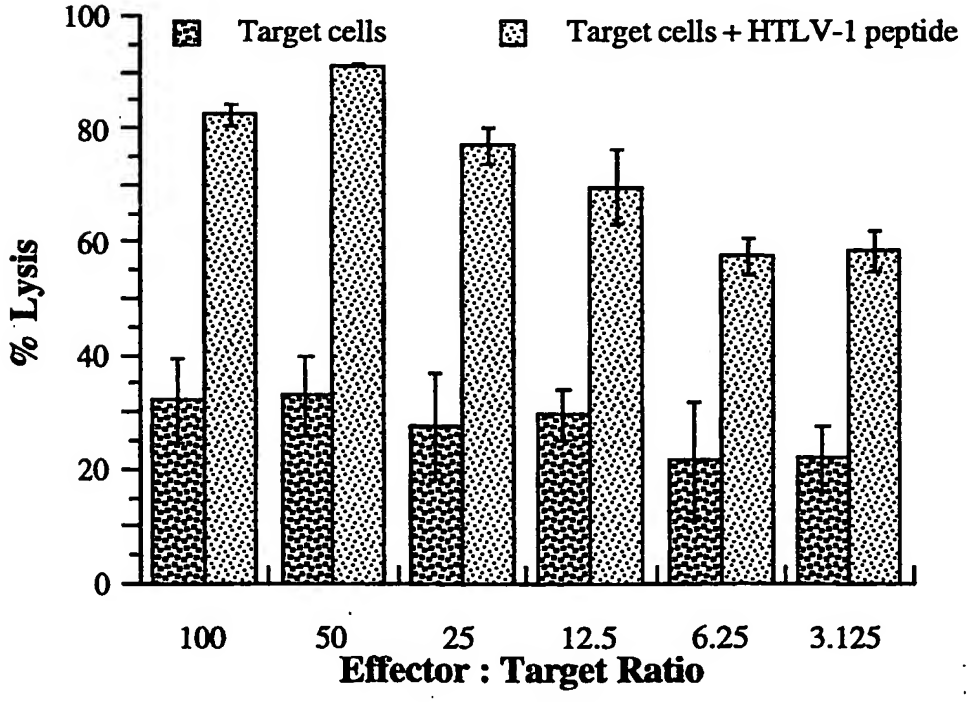

Target cells
Target cells + HTLV-1 peptide
% Lysis
100
80
60
40
20
0
100
50
25
12.5
6.25
3.125
Effector : Target Ratio

Fig. 3B

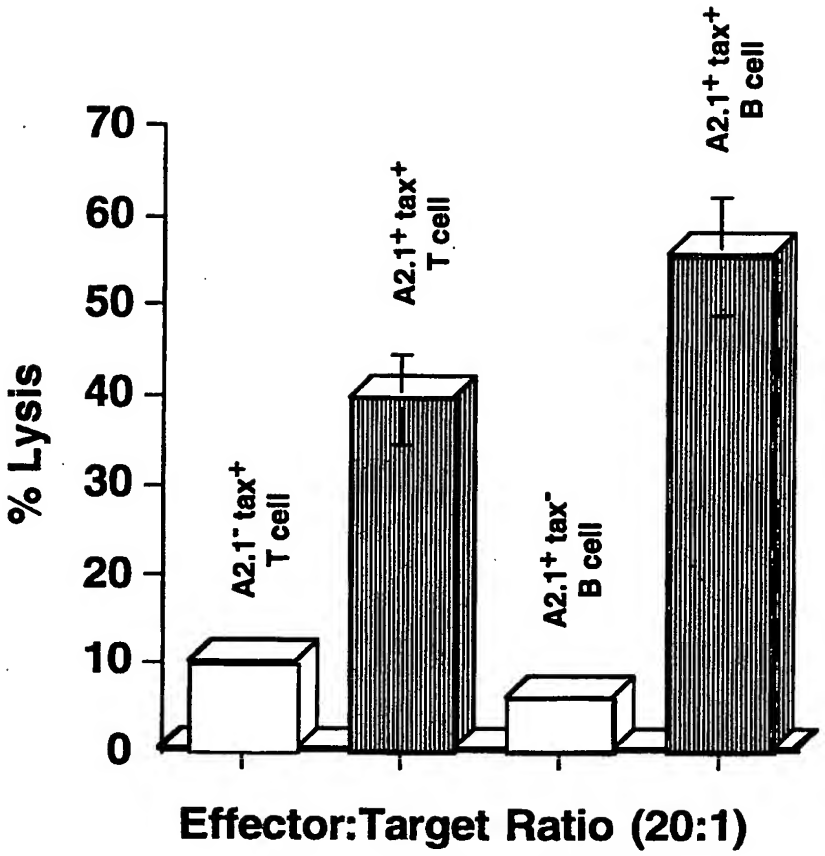

70
60
50
40
30
20
10
0
% Lysis
A2.1⁻ tax⁺ T cell
A2.1⁺ tax⁺ T cell
A2.1⁺ tax⁻ B cell
A2.1⁺ tax⁺ B cell
Effector:Target Ratio (20:1)

Fig. 3C

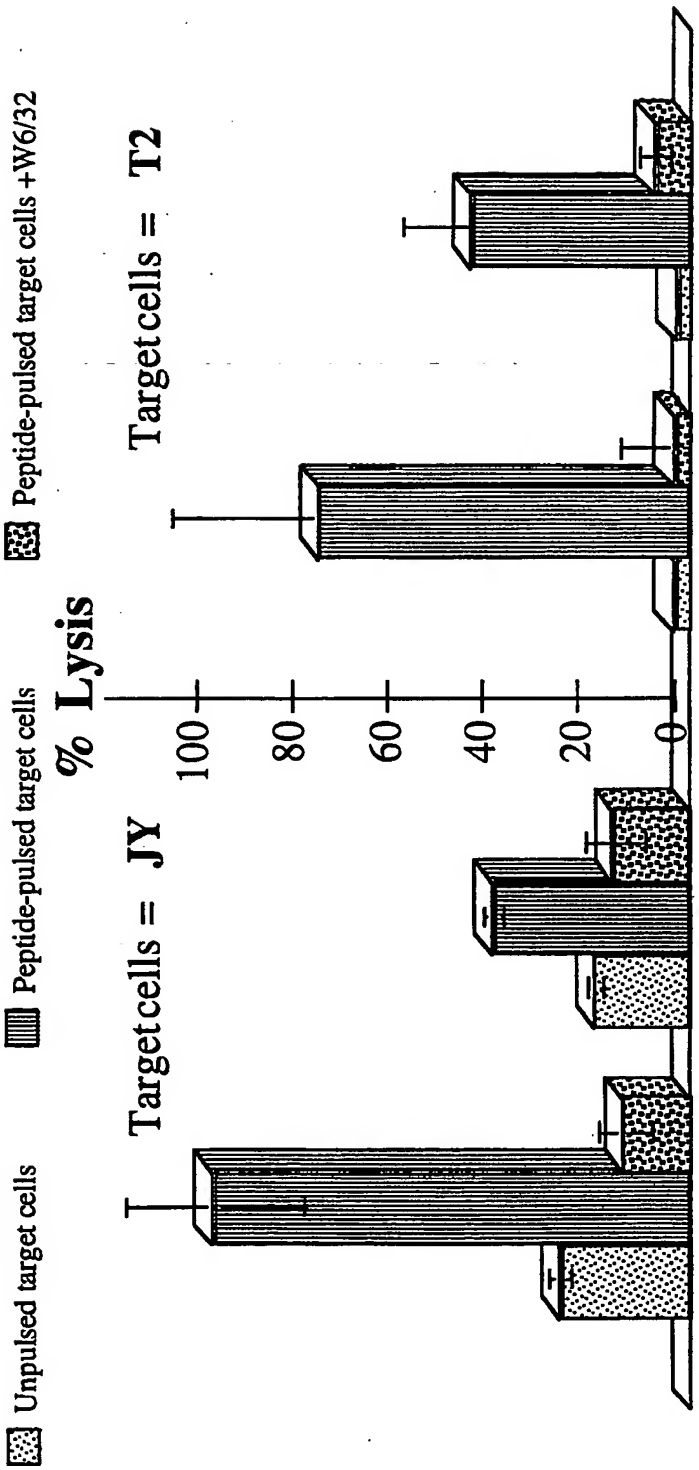
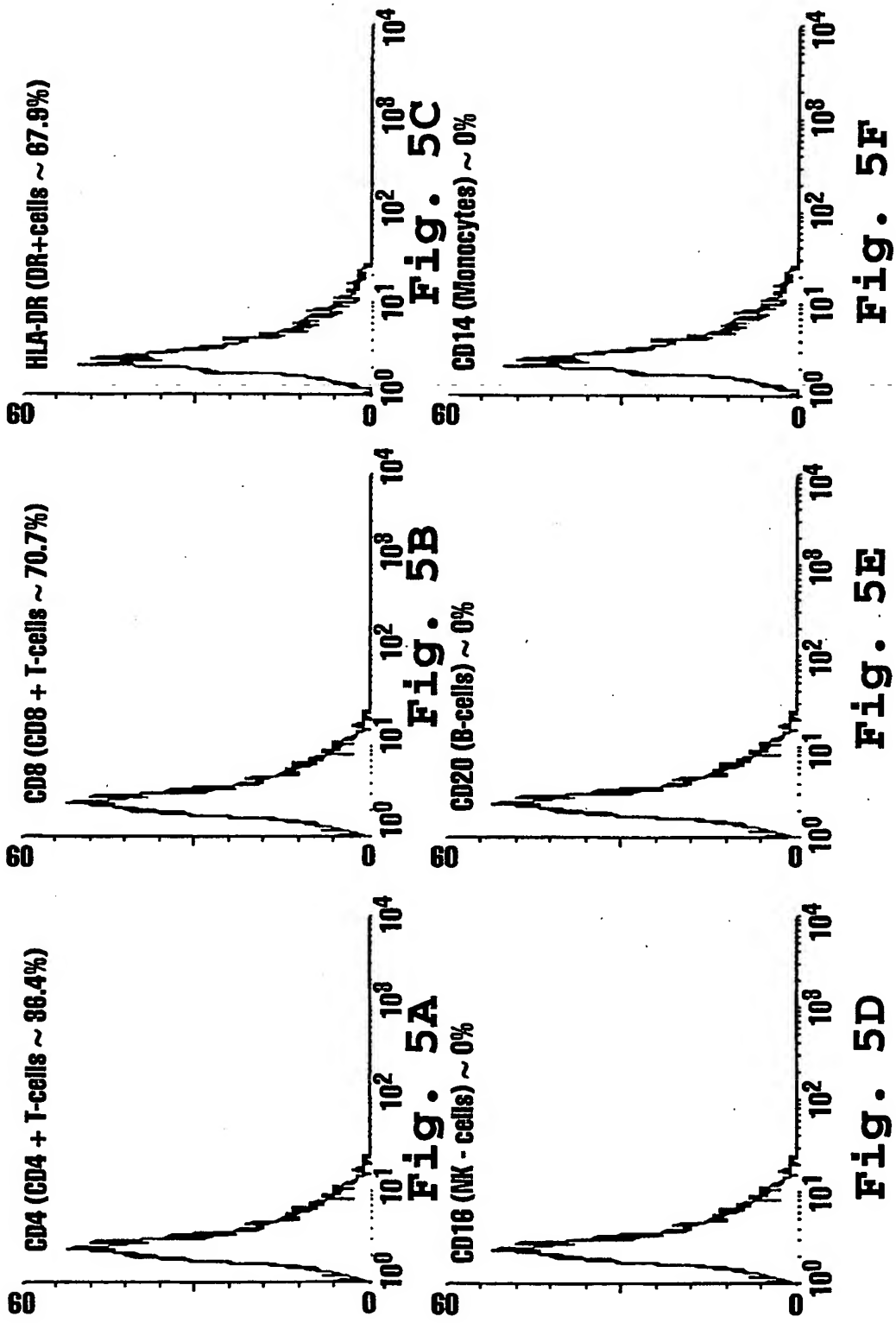

Unpulsed target cells
Peptide-pulsed target cells
Peptide-pulsed target cells +W6/32
% Lysis
100
80
60
40
20
0
Target cells = JY
34:1
17:1
Effector:Target
Target cells = T2
42:1
21:1
Effector:Target

Fig. 4A

Fig. 4B


CD4 (CD4 + T-cells ~ 38.4%)
Fig. 5A
CD8 (CD8 + T-cells ~ 70.7%)
Fig. 5B
HLA-DR (DR+cells ~ 67.9%)
Fig. 5C
CD16 (NK - cells) ~ 0%
Fig. 5D
CD20 (B-cells) ~ 0%
Fig. 5E
CD14 (Monocytes) ~ 0%
Fig. 5F
60
0
10^0
10^1
10^2
10^3
10^4

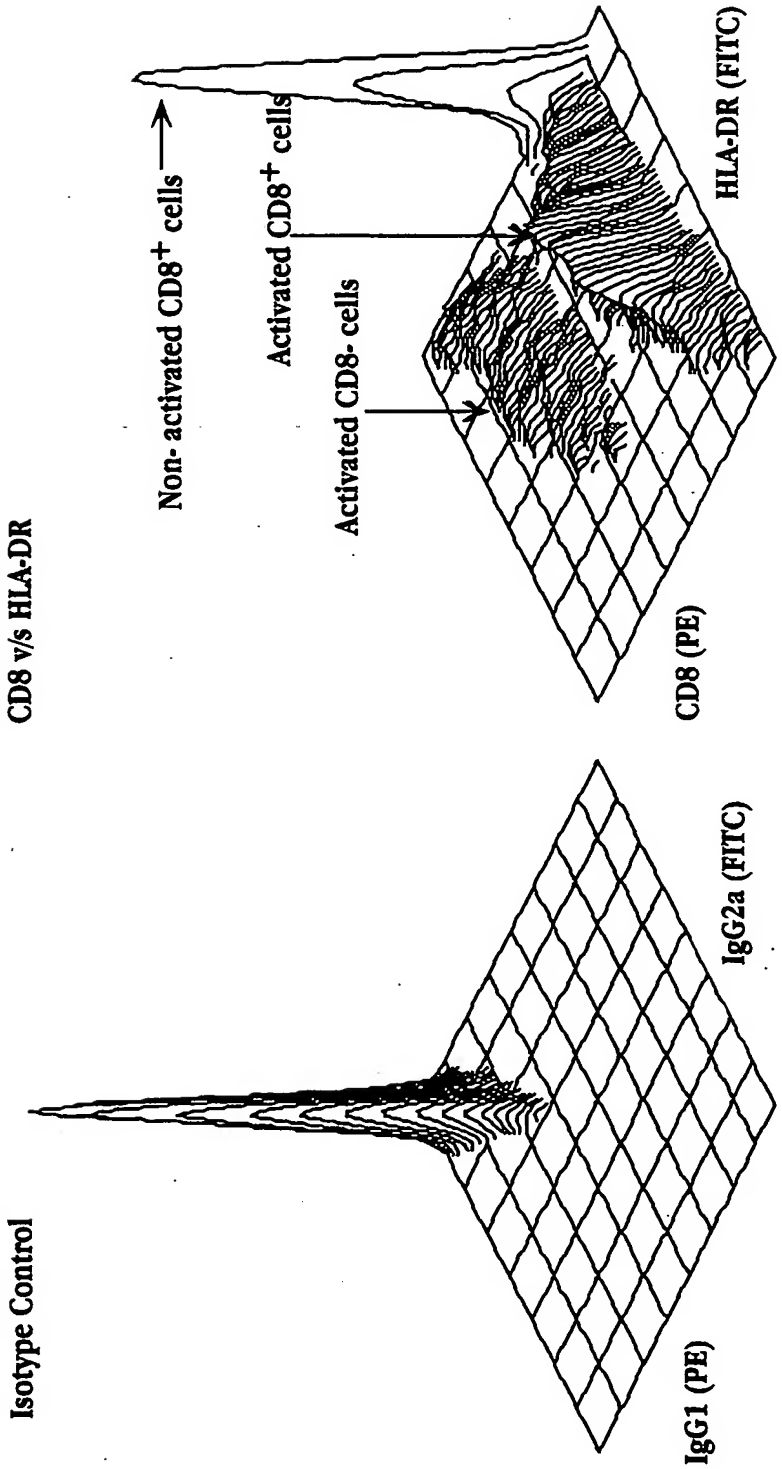

CD8 v/s HLA-DR
Non- activated CD8+ cells
Activated CD8+ cells
Activated CD8- cells
HLA-DR (FITC)
CD8 (PE)
Isotype Control
IgG2a (FITC)
IgG1 (PE)

Fig. 6B

Fig. 6A

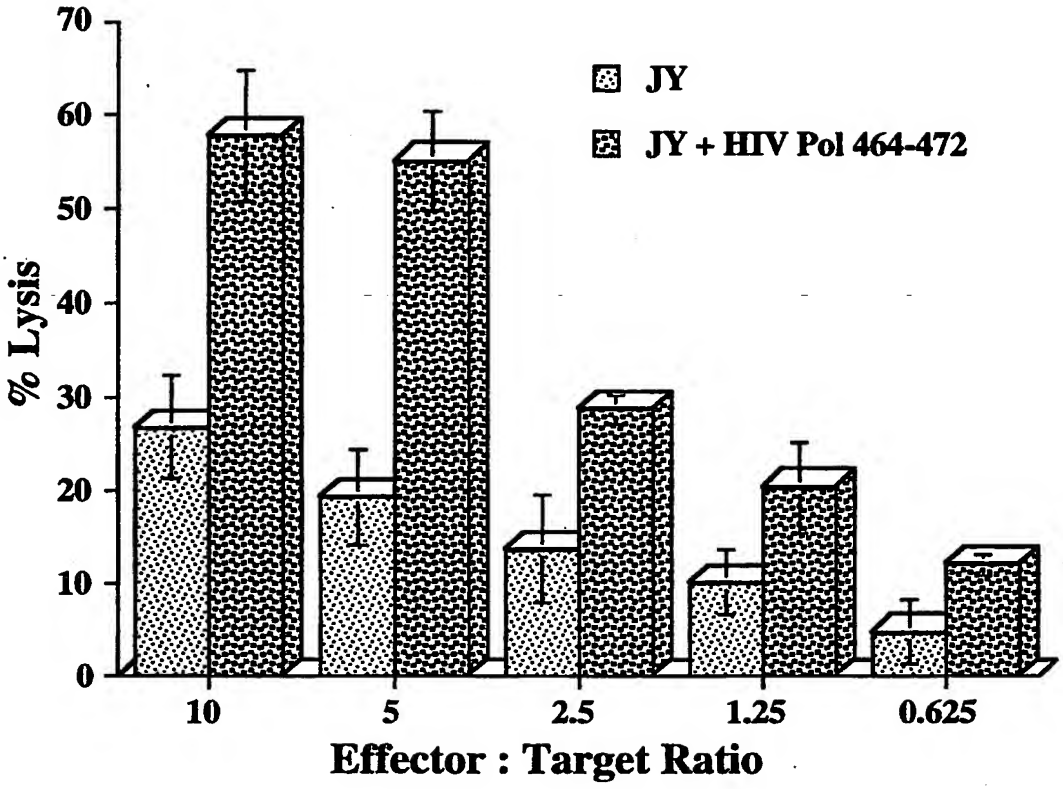

70
60
50
40
30
20
10
0
% Lysis
JY
JY + HIV Pol 464-472
10
5
2.5
1.25
0.625
Effector : Target Ratio

Fig. 7

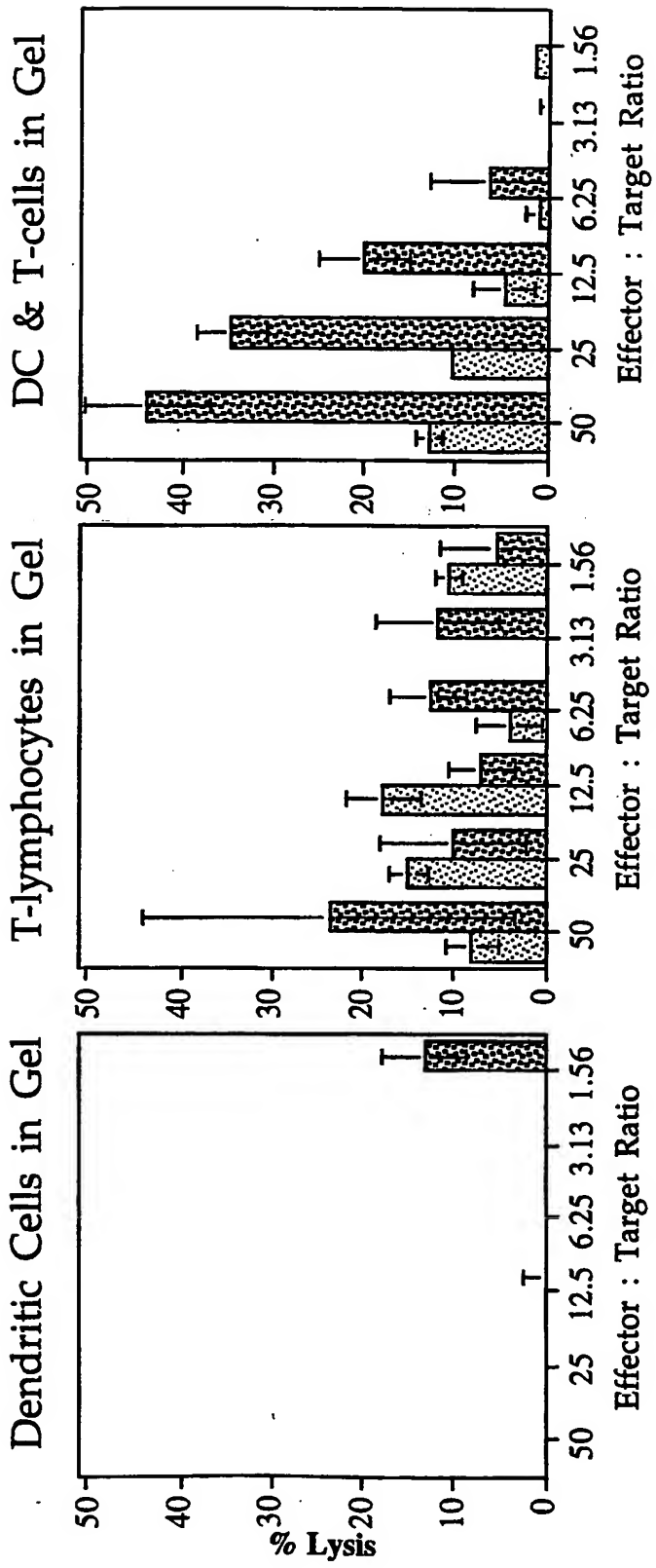


Fig. 8A

Fig. 8B

Fig. 8C

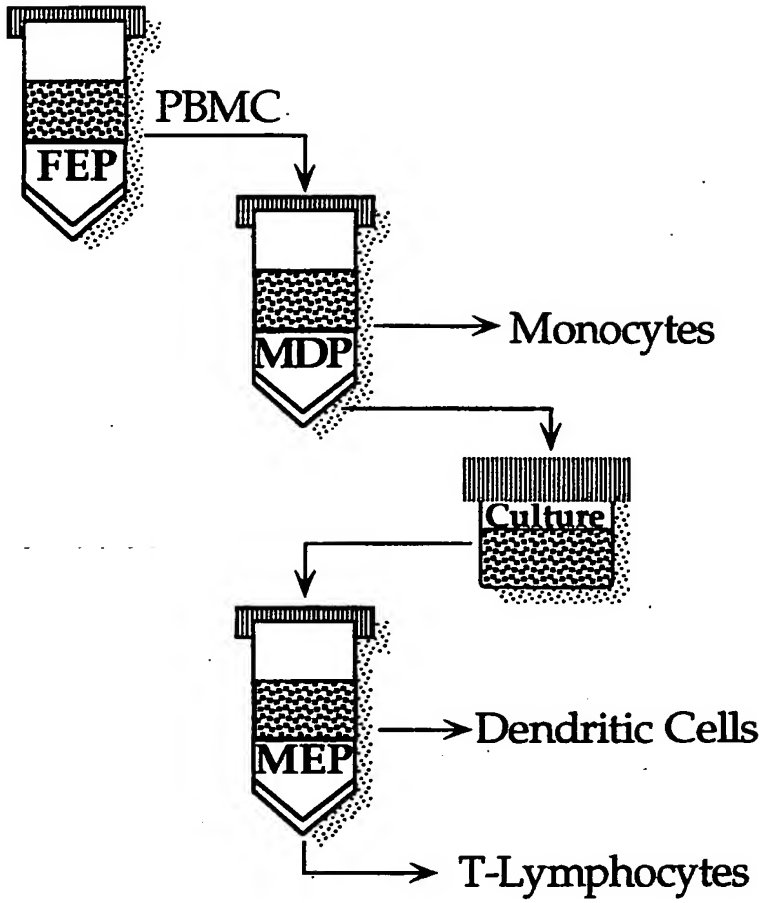

PBMC
FEP
MDP
Monocytes
Culture
MEP
Dendritic Cells
T-Lymphocytes

Fig. 9A

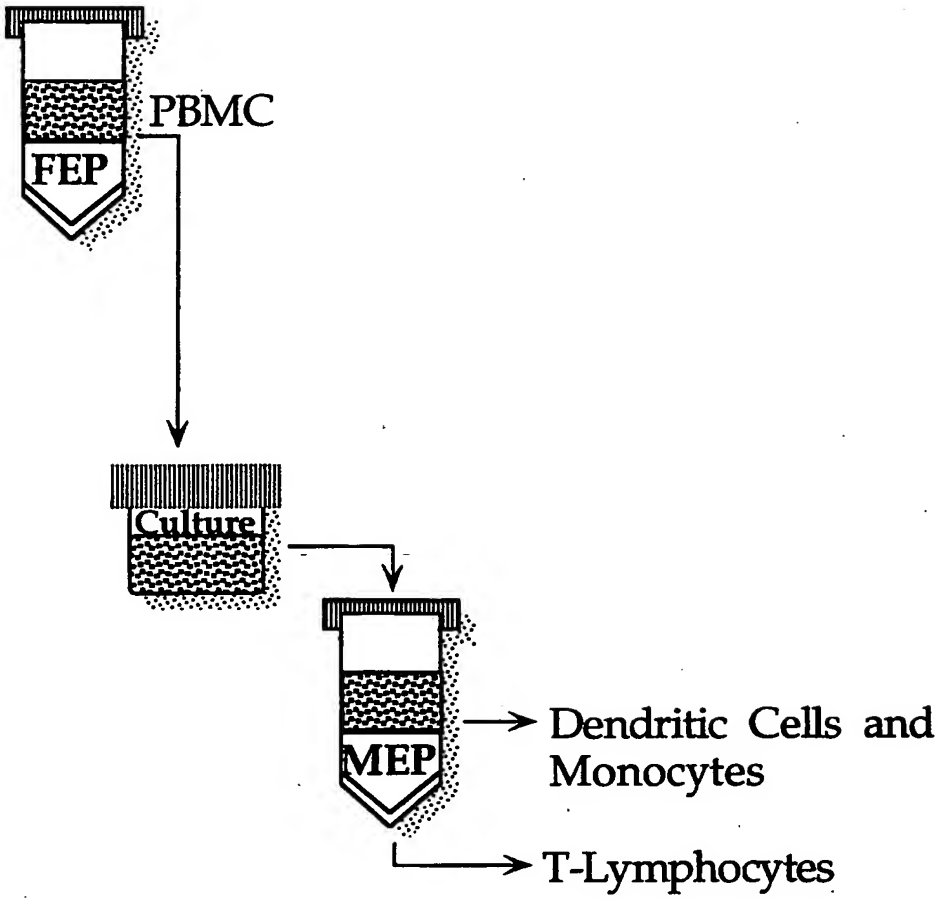

PBMC
FEP
Culture
MEP
Dendritic Cells and Monocytes
T-Lymphocytes

Fig. 9B

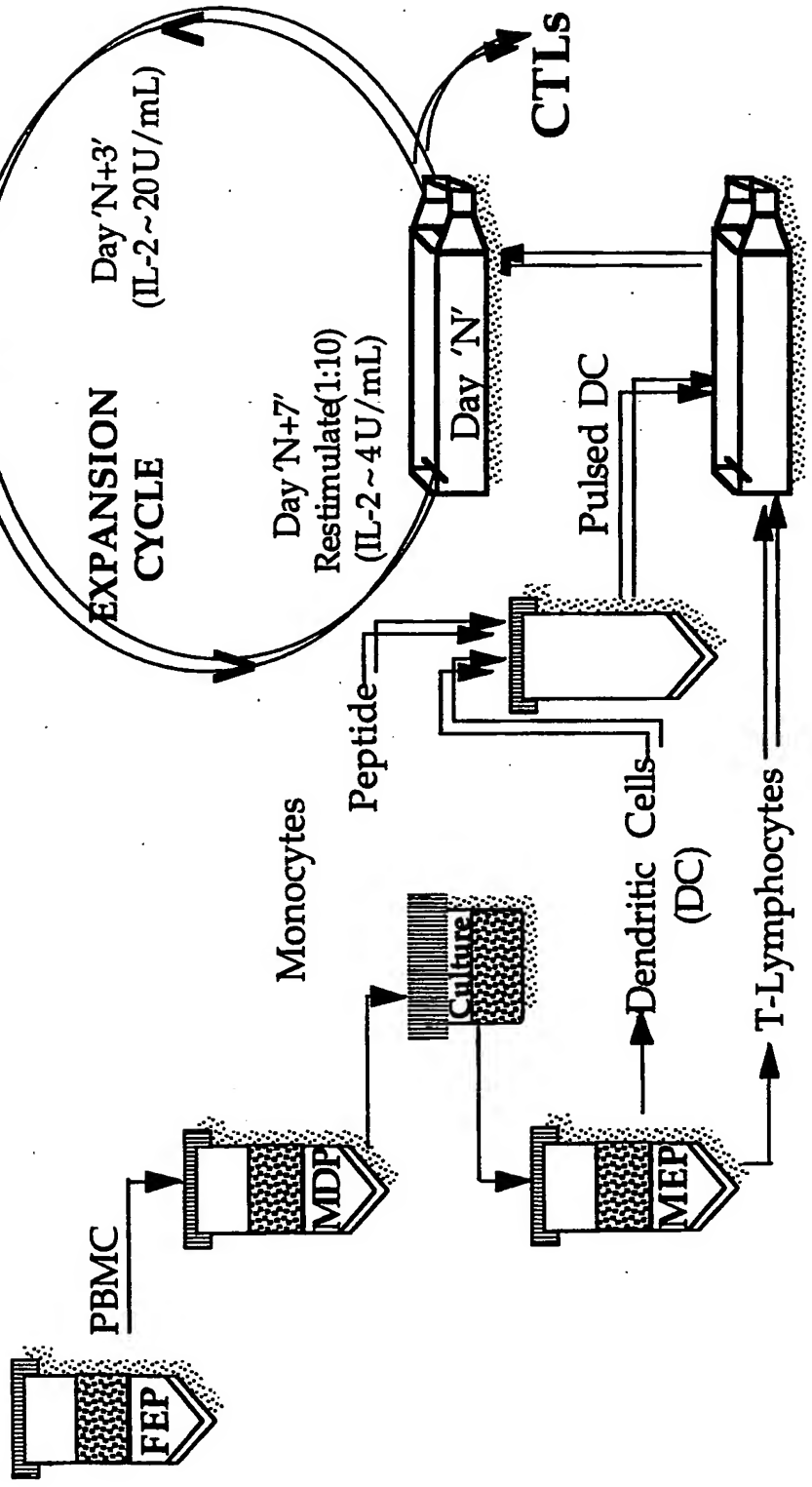

EXPANSION CYCLE
Day 'N+3'
(IL-2~20U/mL)
Day 'N+7'
Restimulate(1:10)
(IL-2~4U/mL)
Day 'N'
CTLs
Pulsed DC
Peptide
Monocytes
Culture
Dendritic Cells
(DC)
T-Lymphocytes
PBMC
MDP
MEP
FEP

Fig. 10

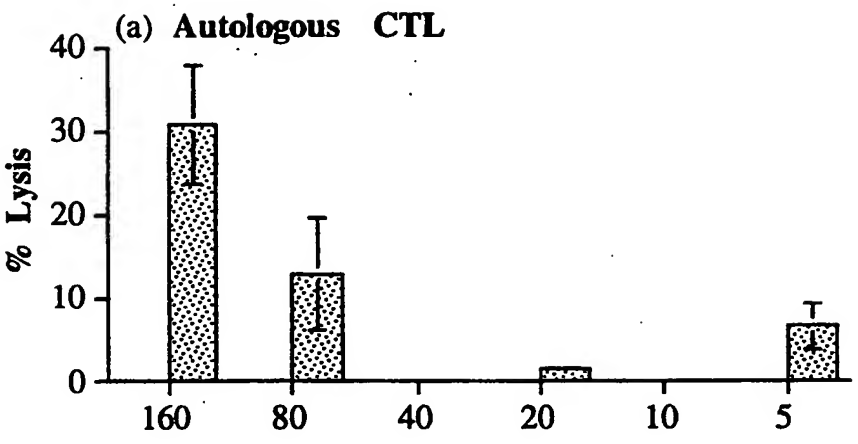


Fig. 11A

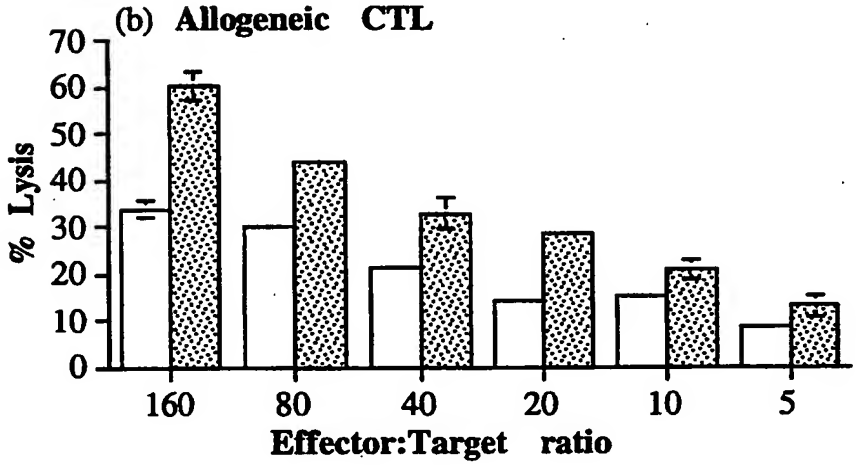


Fig. 11B

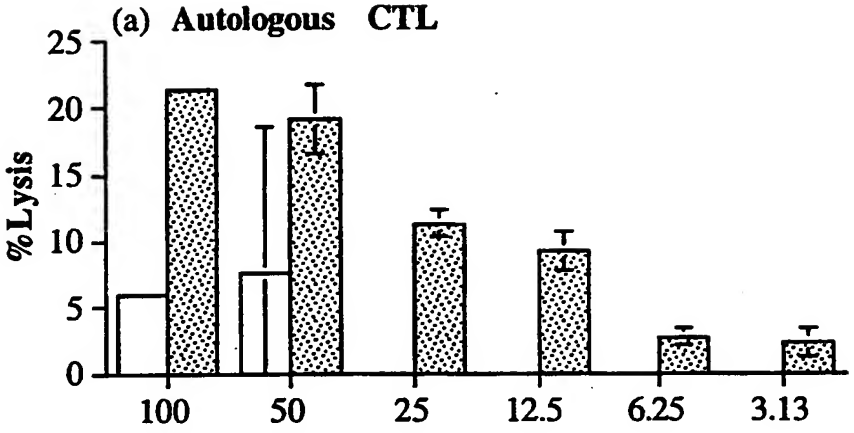


Fig. 12A

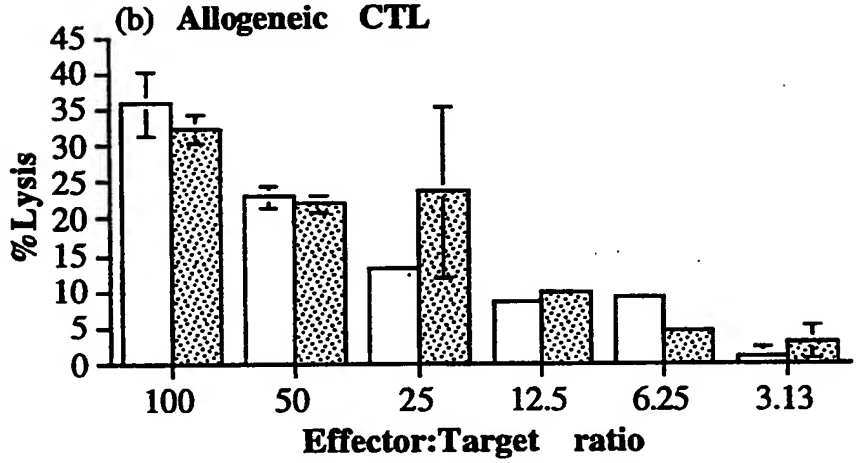


Fig. 12B

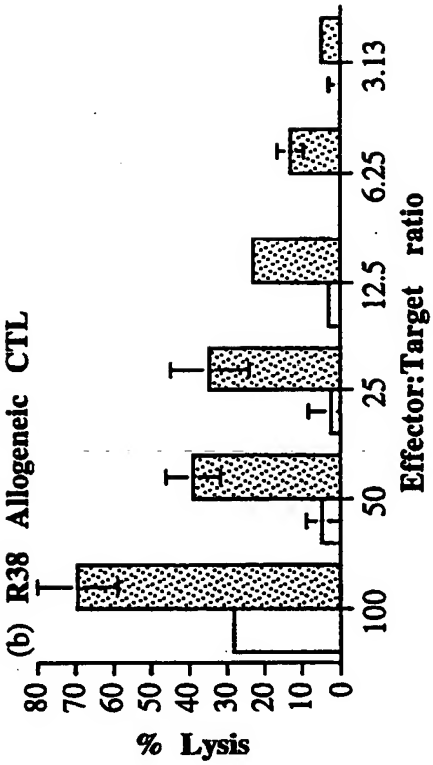


Fig. 13B

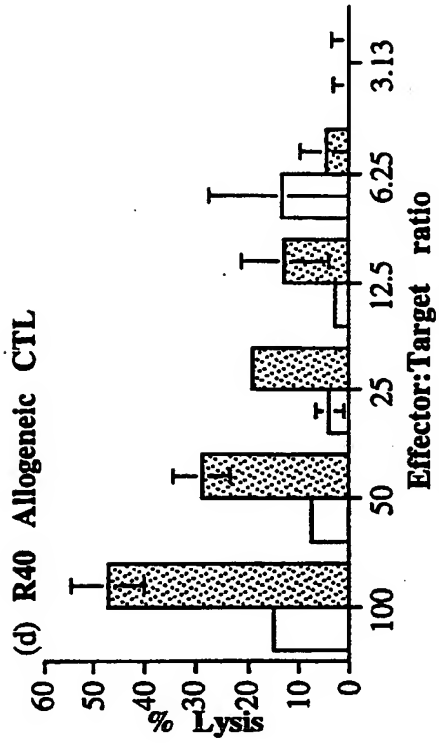


Fig. 13D

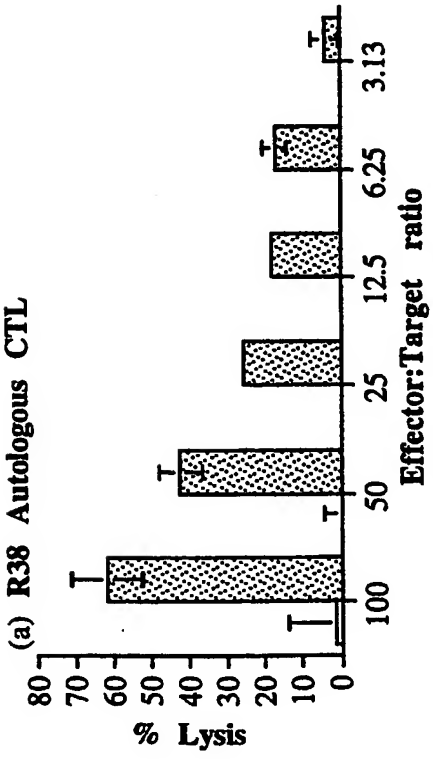


Fig. 13A

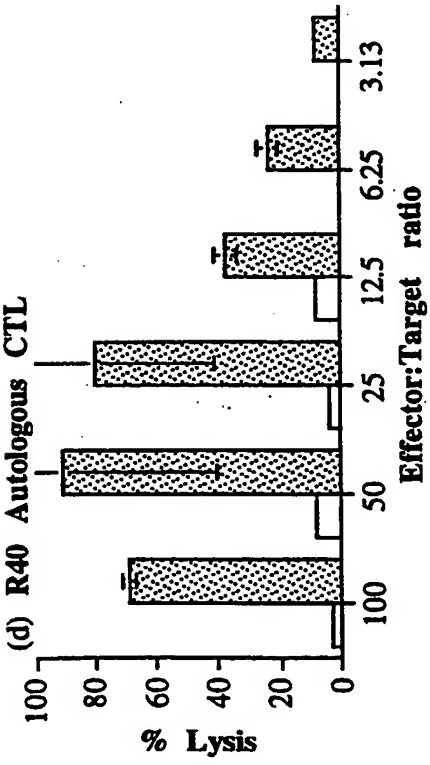


Fig. 13C

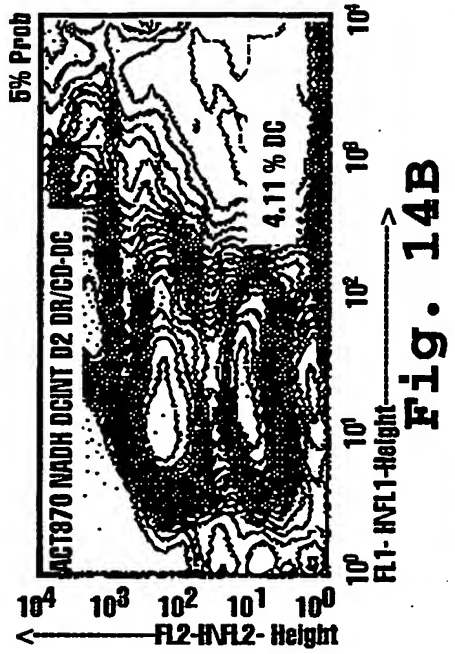


Fig. 14B

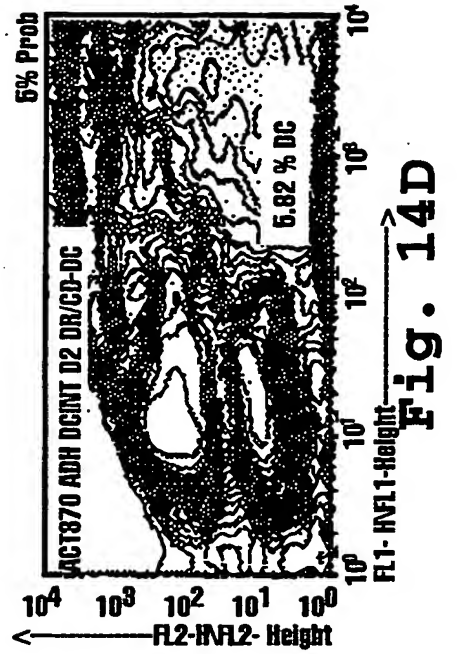


Fig. 14D

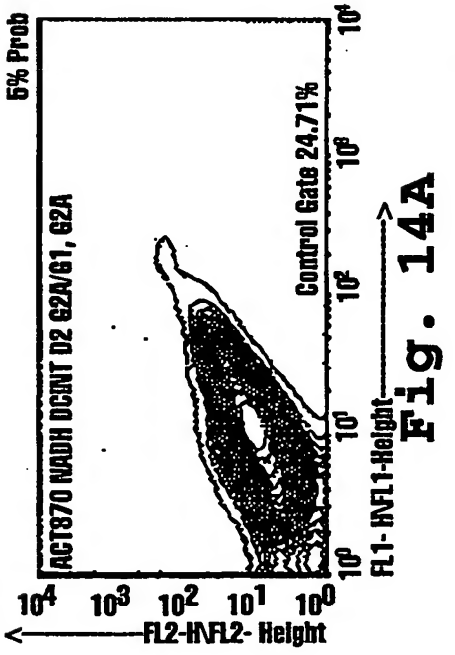


Fig. 14A

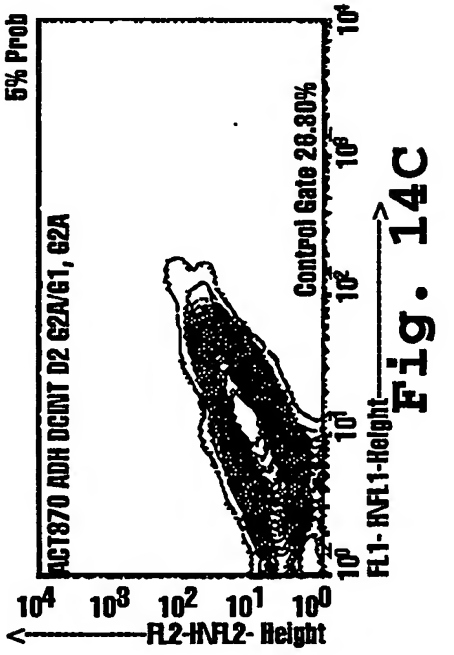


Fig. 14C

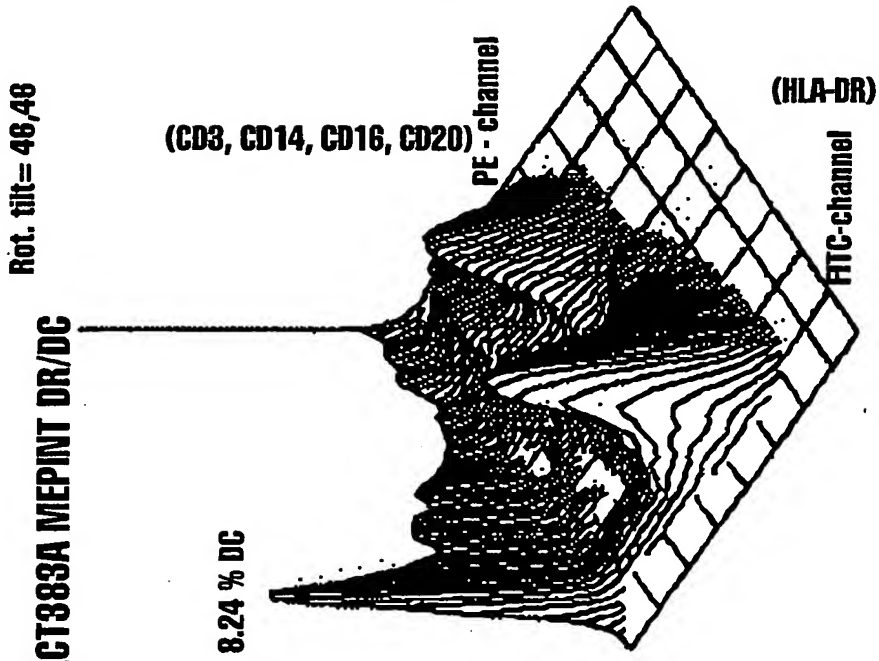


Fig. 15B

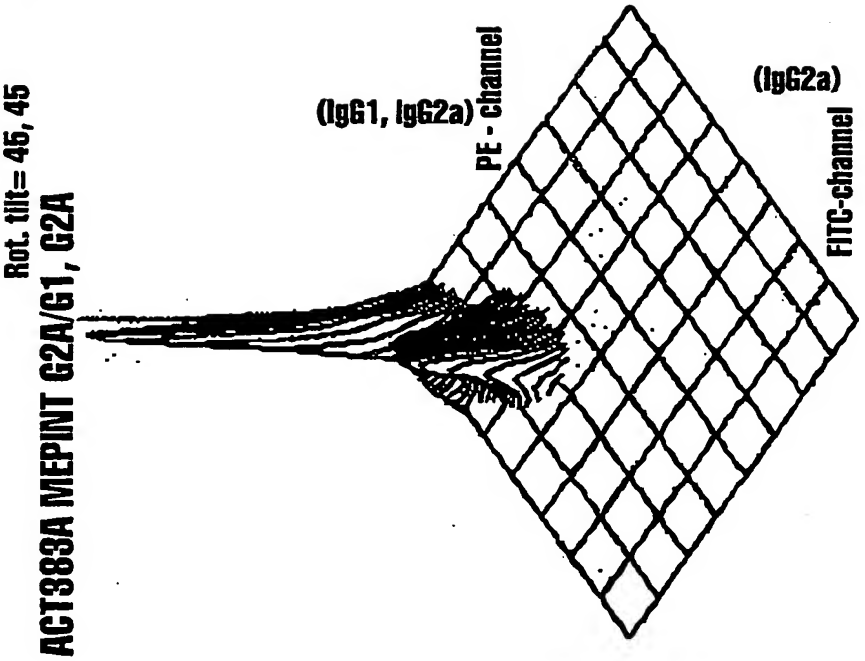


Fig. 15A

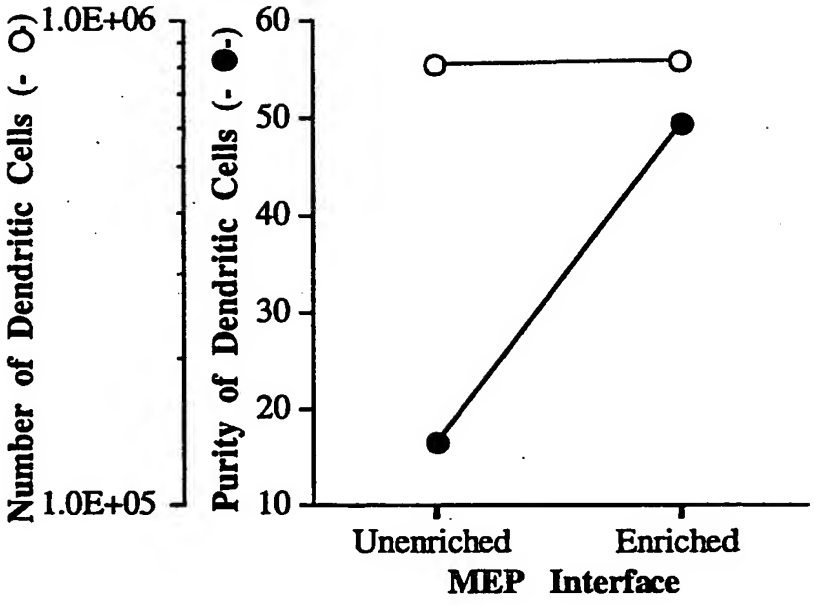

Number of Dendritic Cells (- O-)
1.0E+06
1.0E+05
Purity of Dendritic Cells (- ●-)
60
50
40
30
20
10
Unenriched
Enriched
MEP Interface

Fig. 16

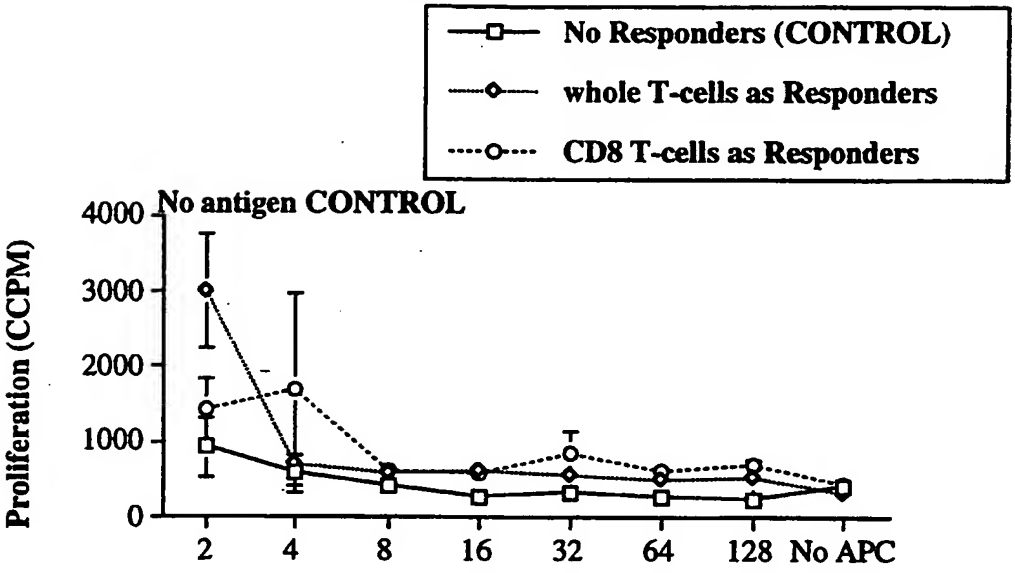


Fig. 17A

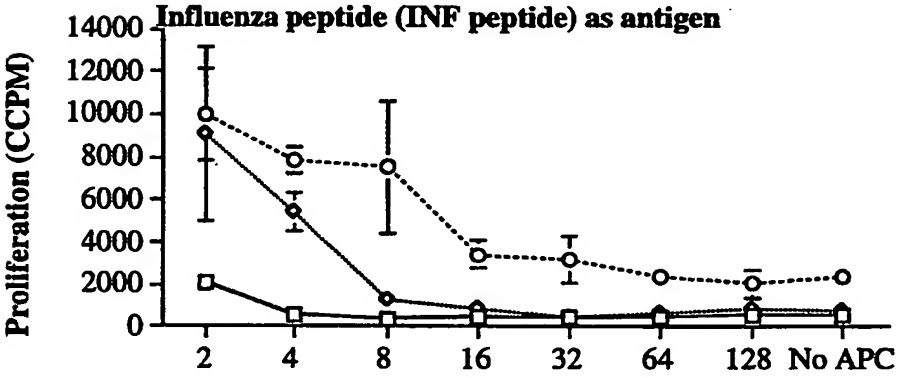


Fig. 17B

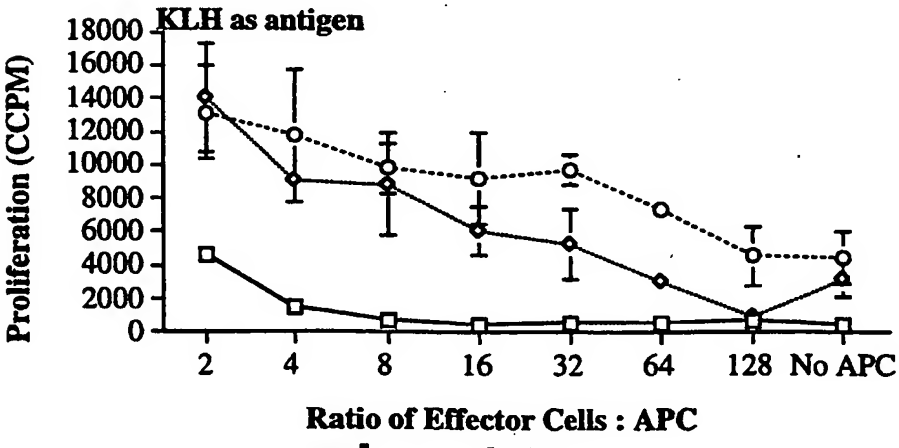


Fig. 17C

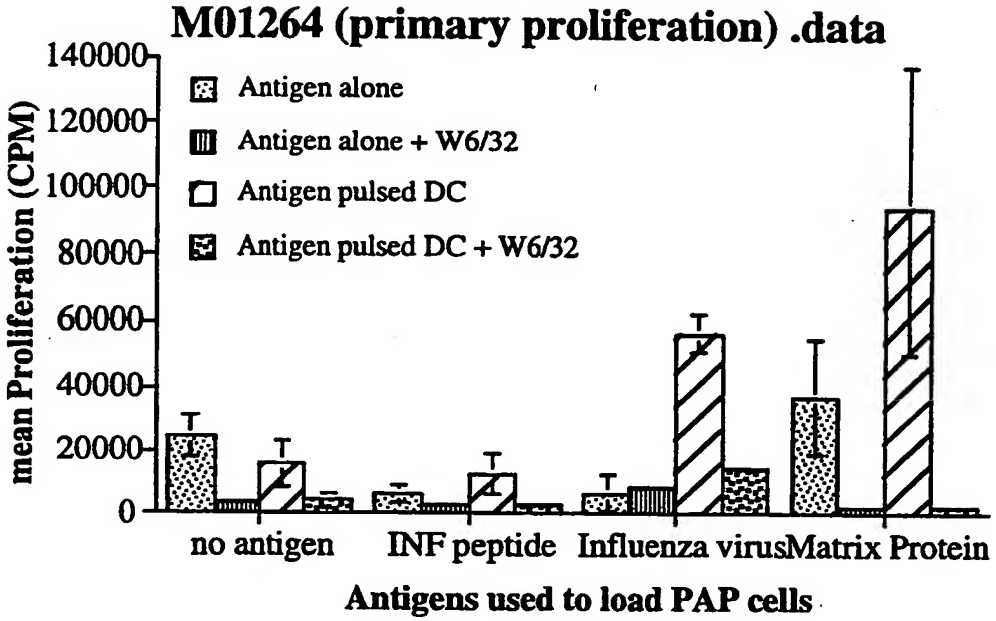

M01264 (primary proliferation) .data
mean Proliferation (CPM)
140000
120000
100000
80000
60000
40000
20000
0
Antigen alone
Antigen alone + W6/32
Antigen pulsed DC
Antigen pulsed DC + W6/32
no antigen
INF peptide
Influenza virus
Matrix Protein
Antigens used to load PAP cells

Fig. 18

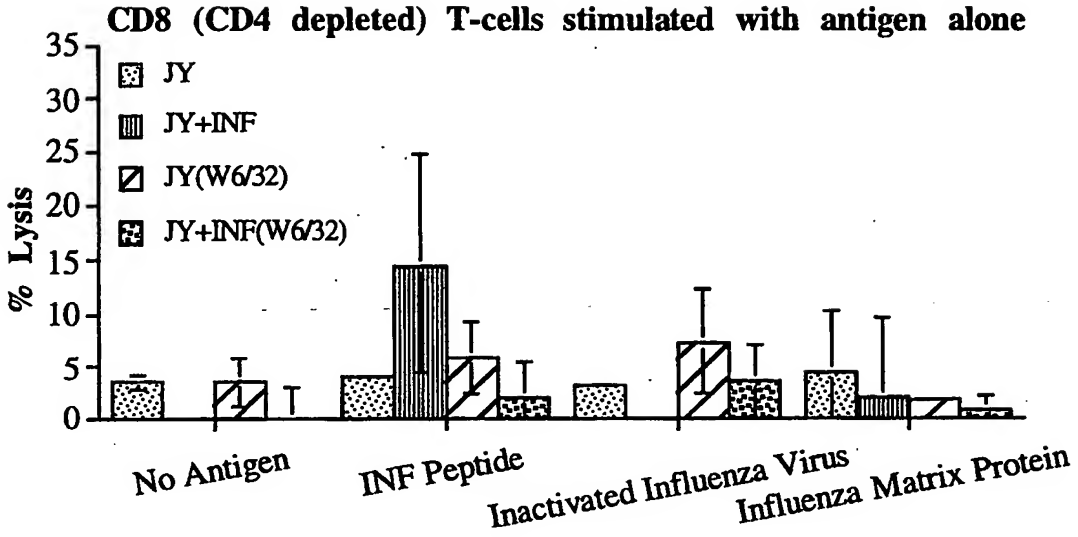

CD8 (CD4 depleted) T-cells stimulated with antigen alone
% Lysis
35
30
25
20
15
10
5
0
JY
JY+INF
JY(W6/32)
JY+INF(W6/32)
No Antigen
INF Peptide
Inactivated Influenza Virus
Influenza Matrix Protein

Fig. 19A

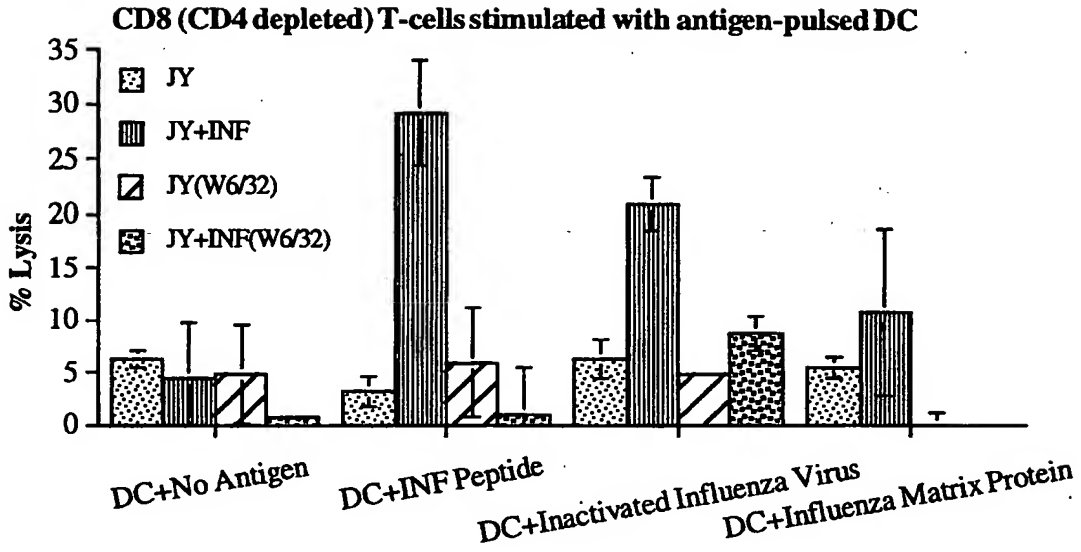

CD8 (CD4 depleted) T-cells stimulated with antigen-pulsed DC
% Lysis
35
30
25
20
15
10
5
0
JY
JY+INF
JY(W6/32)
JY+INF(W6/32)
DC+No Antigen
DC+INF Peptide
DC+Inactivated Influenza Virus
DC+Influenza Matrix Protein

Fig. 19B